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Uncovering HPV awareness, viral integration,
and treatment options in preclinical models

New opportunities to decrease the impact of head and neck cancer: uncovering HPV awareness, viral integration, and treatment options in preclinical models

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Chapter 1

General introduction and outline of this thesis

Head and neck cancer

In 2020, approximately 930,000 new cases of head and neck cancer were reported worldwide, representing 5% of all cancer diagnoses. Approximately 470,000 deaths could be attributed to head and neck cancer in the same year, indicating an overall mortality rate of 50%. In The Netherlands, over 5,600 new cases of head and neck cancer were reported in 2020.[1] Head and neck squamous cell carcinoma (HNSCC) is the most prevalent histological type of head and neck cancer, responsible for 95% of cases. These tumors originate from the squamous epithelial cells forming the mucosal lining of the upper aerodigestive tract. Although originating from the same cell type, HNSCC present a heterogeneous group of tumors. This may be explained by the diverse and complex anatomical locations in which tumors develop, different aetiologies, and a broad range of genetic and molecular changes driving carcinogenesis.[2]

The major anatomical locations in which these tumors arise are the oral cavity, nasal cavities, nasopharynx, oropharynx, hypopharynx, and larynx. The burden of HNSCC in general as well as the distribution of anatomical sublocations varies across countries/regions and is mainly correlated with exposure to tobacco-derived carcinogens and/or heavy alcohol consumption.[3] Cancers of the lip and oral cavity are highly frequent in South Central Asia (e.g. India, Sri Lanka, Pakistan) and Melanesia (e.g., Papua New Guinea), probably associated with the carcinogenic effects of betel nut chewing.[4] Oropharyngeal squamous cell carcinomas (OPSCC) are increasingly linked to infections with mucosal oncogenic Human Papillomaviruses (HPV), primarily HPV16[3,5] HPV-related tumors are a distinct tumor entity, exhibiting distinct differences from HPV-negative HNSCC in clinical presentation and molecular profiles (Table 1.1).[2,6,7] Therefore, in the next paragraphs of this introduction, risk factors, disease characteristics, genetic changes, and deregulated molecular pathways in both HPV-positive and HPV-negative HNSCCs are described.

Viral carcinogenesis in HPV-positive HNSCC

Infection with high-risk HPV is an increasingly common risk factor for the development of OPSCC, especially in so-called 'high income' countries.[8-11] The portion of HPV-associated OPSCC in The Netherlands increased from 5.1% in 1990 to 30-50% in recent years.[12-15] In comparison to patients with HPV-negative OPSCC, patients with HPV-associated OPSCC are younger, more often male, have a higher socioeconomic status, have more lifelong (oral) sexual partners, and are less likely to have a history of tobacco

and alcohol use (Table 1.1).[8,16,17] HPV-positive tumors generally present as smaller (asymptomatic) tumors, often with regional lymph node metastases and sometimes with presentation of neck metastases from an occult primary tumor.[18] Despite this peculiar clinical presentation, HPV-associated tumors generally have a better prognosis compared to HPV-negative tumors, independent of treatment modality (Table 1.1).[19,20]

Table 1.1 Clinicopathological characteristics of HPV-positive and HPV-negative HNSCC.

	HPV-positive	HPV-negative
Anatomical site	Oropharynx	All sites
Incidence	Increasing	Decreasing
Risk factors	(Oral) sexual behavior, immunosuppression	Smoking, excessive alcohol consumption
Age	Younger (<60)	Older (>60)
Socioeconomic status	Higher	Lower
Tumor size	Early T stage (T1-2)	Variable
Lymph node involvement	Advanced	Variable
Mutational burden	Low	High
5-year survival	Good (80-90%)	Poor (40-50%)

Next to distinct clinical characteristics, HPV-associated tumors exhibit differences in gene expression and mutational profiles compared to HPV-negative HNSCC, underscoring the unique biology of this disease.[21] Most of these tumors arise from the crypts of the palatine and lingual tonsils, where HPV infects the basal cell layer of the stratified epithelium.[21] The majority of HPV-positive OPSCC is caused by infection with HPV16 (>90%). Other high-risk HPV types, including HPV18, HPV31, HPV33, and HPV52 are detected in a small percentage of patients.[22,23] HPVs are double-stranded, circular DNA viruses, with a genome of approximately 8 kilobases (kb). Their genome consists of 8 protein coding genes, including 6 early genes (E1, E2, E4, E5 E6, E7), and 2 late genes (L1, L2). Upon infection of the basal cells, expression of E1 and E2 facilitates viral DNA replication from episomes. When replicating cells move into the parabasal layers, E6 and E7 expression suppresses differentiation and causes cells re-entering the cell cycle. HPV-mediated carcinogenesis is largely driven by the viral oncogenes E6 and E7. These proteins target many cellular proteins resulting in malignant transformation of the host cell.[23-27] The E6 protein binds and promotes proteasomal degradation of p53, preventing cell death.[28] The E7 protein binds to the key cell cycle regulator RB1, promoting proteasomal destruction of this protein, followed by activation of the E2F family of transcription factors. These transcription factors drive the cell cycle beyond the G1-S checkpoint, resulting in re-entry in the cell cycle (Figure 1.1). This disruption of RB1 by E7 leads to a feedback upregulation of the $p16^{INK4A}$ protein, which serves as a surrogate marker for HPV positivity in routine diagnostics.

Mutations in tumor suppressor genes are uncommon in HPV-positive HNSCC, in contrast to HPV-negative tumors.[7] Nevertheless, chromosomal losses of regions 14q32 and 9q have been observed in HPV-positive tumors, containing the tumor necrosis factor receptor-associated factor 3 (*TRAF3*) and ataxia telangiectasia mutated (*ATM*) genes, respectively. Molecular alterations in the genes of the PI3K/Akt/mTOR pathway are the most common genetic changes in HPV-positive tumors. These changes include mutations and amplifications of *PIK3CA,* encoding the catalytic subunit of phosphoinositide-3-kinase (PI3K), often resulting from gain of the chromosomal 3q region.[2,29,30] The squamous transcription factor TP63 is also located in this 3q region and therefore found to be overexpressed in a subset of HPV-positive HNSCCs, leading to impaired differentiation.[7] In addition, inactivation of NOTCH signaling is often observed, by downregulation of p53 and TP63 (Figure 1.1).[7,31] Furthermore, a specific mutational profile is observed in HPV-positive tumors, caused by high cytosine deaminase activity associated with the apolipoprotein B mRNA editing enzyme catalytic (APOBEC) enzyme family. This is induced as response to viral infections and preferably leads to cytosine-to-thymidine (C>T) mutations.[7,32-34]

During a persistent HPV infection, the integration of the viral genome into the host genome is an additional genetic event that could take place. It is thought that viral integration requires both viral and host DNA breakage, e.g., induced by reactive oxygen species, inflammation, or APOBEC mediated mutagenesis.[35-38] The exact percentage of HPV-positive OPSCC with integration is under debate and is highly dependent on HPV integration detection technique. Recent studies suggest that 50% of HPV-positive OPSCC harbor HPV integration sites in the human genome.[39-43] However, it is still questioned whether HPV integration is associated with distinct biological consequences and patient outcomes.

Genetic landscape of HPV-negative HNSCC

Tobacco consumption and excessive alcohol consumption are the key risk factors for the development of HPV-negative HNSCC. Tobacco-derived carcinogens undergo metabolic modification, resulting in reactive metabolites. These metabolites form covalent DNA adducts, which, if not repaired properly, lead to mutations and other genetic abnormalities.[21] Excessive alcohol consumption acts as an independent risk factor for the development of HNSCC.[44-46] Acetaldehyde, its major metabolite, is suggested to disrupt DNA synthesis and repair, bind proteins, and form stable DNA adducts, amongst others.[44] Furthermore, ethanol may act as a solvent for tobacco-derived carcinogens,

increasing the exposure of epithelial cells to these substances, which increases the risk of HNSCC synergistically.[47] The accumulation of multiple (epi)genetic changes in key tumor suppressor genes or oncogenic signaling pathways is associated with the onset and progression of HNSCC.[48,49] In HPV-negative HNSCC, these changes affect multiple carcinogenic pathways, predominantly cell cycle regulation and cell survival (Figure 1.1).

Early genetic events often include loss or gain of chromosomal regions. For example, loss of the 9p21 (including *CDKN2A* and *CDKN2B)*, 3p21, and 17p13 regions (including *TP53*), or amplification of 11q13 and 11q22 regions (including *BIRC2* and *FADD*) are observed.[7,50] In addition to chromosomal alterations, somatic mutations are common in HPV-negative HNSCC. Detailed genomic characterization of 279 HNSCCs by The Cancer Genome Atlas (TCGA) revealed frequent mutations in tumor suppressor genes *TP53* (72%), *CDKN2A* (22%), *FAT1* (23%), *NOTCH1* (19%), *KMT2D* (18%), *NSD1* (10%), and *TGFBR2* (4%).[7] Key regulators of oxidative stress, *NRF2* and *KEAP1*, are also commonly mutated in HPV-negative HNSCC. Mutations in *HRAS* and *CASP8* are observed in a relatively small percentage of HPV-negative HNSCC. Amplifications of the oncogene *MYC* and receptor tyrosine kinases (including *EGFR*, *HER2*, and *FGFR1*) are often found.[51-53] Also, hypermethylation of *CDKN2A* is common, describing an additional downregulating mechanism to homozygous deletions and mutations of this gene.[54,55] Interestingly, *PIK3CA* is the only oncogene that is found to be frequently mutated in HPV-negative HNSCC (14%).[7,29,56] Among signaling pathways driving tumorigenesis, the PI3K/Akt/mTOR pathway is the most frequently altered as a result of mutations, gene amplifications, or loss of function of the negative regulator phosphatase and tensin homologue (PTEN).[7] Alterations in the genes of the PI3K/Akt/mTOR pathway are common in both HPV-negative and HPV-positive tumors, making this pathway a driver for HNSCC in general. Similarly, inactivation of NOTCH and upregulation of TP63 is observed in both HPV-negative and HPV-positive HNSCC (Figure 1.1).[7]

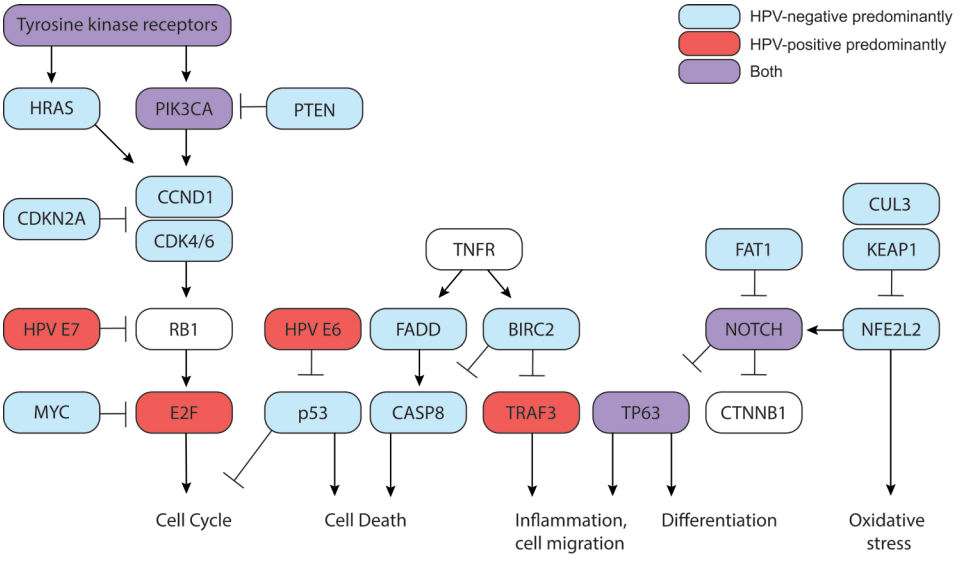

Figure 1.1 Overview of key affected pathways, factors, and functions in HPV-negative HNSCC, HPV-positive HNSCC or both. Pathway alterations include homozygous deletions, amplifications, somatic mutations, or protein degradation by viral oncogenes E6/E7.

Treatment of HNSCC

The treatment approach for HNSCC is guided by anatomical site, disease stage, disease characteristics, functional considerations, health condition, and patient wishes. Various subsites of HNSCC are treated differently, but within subsites the heterogeneous nature of tumors (e.g., genetic profile, HPV status) does not lead to significant differences in treatment to date.

In patients with small primary tumors with no or limited lymph node involvement, single modality treatment is recommended. In The Netherlands, most oral cavity tumors are treated with surgery, whereas radiotherapy is commonly applied for pharyngeal tumors. Small laryngeal tumors are treated by laser surgery or radiotherapy. Selective neck dissection or (prophylactic) neck radiotherapy is considered when there is (an increased risk for) limited lymph node involvement. In patients with oral cavity tumors without neck node metastases, but increased risk of occult metastases, sentinel node procedures are recommended.[57] Advances in minimally invasive surgery, including transoral robotic or laser resection, have extended the indications for primary surgical treatment.[58,59] In HPV-positive tumors, de-intensification of treatment has been studied in several trials,

but these were not able to proof that de-intensification of treatment is equivalent to standard treatment plans so far.[60,61] More de-intensification trials are ongoing of which the results are awaited.[62]

For more advanced tumors, multimodal treatment approaches are recommended. In The Netherlands, surgical resection and (selective) neck dissection, followed by (chemo)radiotherapy is recommended for advanced oral cavity cancer and laryngeal tumors with massive cartilage infiltration or severely disturbed function. For pharyngeal or larger laryngeal tumors without massive cartilage infiltration or function loss, (cisplatin-based) chemoradiotherapy is the standard of care.[57] For older patients or patients in a poor general condition, cisplatin-based chemotherapy can be replaced by the EGFR inhibitor cetuximab. Patients with local or locoregional recurrence and/or metastases of tumors treated with (chemo)radiotherapy can be treated with salvage surgery if the recurrence is resectable and the overall condition of the patient is sufficient. Alternatively, systemic therapy could be applied. Re-irradiation can be considered when the primary treatment has been finished more than one year ago, however, re-irradiation is challenging due to the limited tolerance of normal tissues, and often leads to (severe) side effects. Furthermore, treatment with immune checkpoint inhibitors (pembrolizumab, nivolumab), approved as first-line treatment in recurrent/metastatic disease can be considered, either or not in combination with chemotherapy.[57,63-65]

Despite improvements in therapeutic options, the prognosis of HNSCC remains poor and (long-term) side effects of therapy are still substantial. There is no doubt that prevention is the best option to reduce the impact of cancer. In HPV-driven cancers, vaccination programs offer the possibility to prevent the development of cancer. HPV vaccination has been offered to girls for approximately two decades, which has led to decreased rates of cervical cancer in countries where HPV vaccination is widely available.[66,67] Several countries, including The Netherlands, have now extended vaccination programmes to boys, but it is estimated to take at least 20-30 years before the benefits of gender-neutral vaccination will be demonstrated on incidence rates of OPSCC.[68,69] Furthermore, vaccination rate within the population strongly influence the effect of the vaccination programs.

Clinical challenges

Widespread knowledge about risk factors and public education programs are prerequisites for lifestyle adaptation and a significant reduction of the risk for head and

neck cancer. Whereas the carcinogenic effects of smoking and alcohol consumption are generally well known, the recognition of HPV as a risk factor for OPSCC seems to be less recognized.[68,70] This gap in knowledge is, amongst other factors, responsible for the suboptimal HPV vaccination uptake and missed opportunities in terms of disease prevention.[23,71] Increasing HPV awareness among the general population, but also among health-care providers, is essential to increase vaccination rates, to decrease cancer incidence, and to improve early detection.

In addition, large efforts in clinical care and research have been made in the last decades, aiming to improve outcomes for HNSCC patients. However, survival rates have hardly increased and especially HPV-negative HNSCC still have a poor prognosis. Furthermore, short -and long-term treatment-related side effects are still substantial. These include mucositis, pain, loss of normal voice or impaired speech, swallowing difficulties, salivary gland dysfunction, fibrosis, osteoradionecrosis, dental problems, shoulder dysfunction, lymphedema, and cosmetic changes.[72,73] As mentioned before, standard of care for advanced stage disease is still based on surgery and/or radiotherapy with platinum-based chemotherapy, which was already approved by the FDA in 1978.[74] Despite this, 50-60% of patients with advanced stage HNSCC develop recurrence or distant metastases.[3] Recent advances in the treatment of HNSCC are the approval of the EGFR inhibitor cetuximab and the immune-checkpoint inhibitors nivolumab and pembrolizumab. However, cetuximab treatment shows a limited survival benefit, only demonstrated in patients with contraindications for chemotherapy or in recurrent/metastasized disease.[75] Immune checkpoint inhibitors have shown to improve survival in recurrent/metastasized disease, but the overall response rate is only 20% and survival is increased by a few months.[76] Therefore, there is an urgent need for improved treatment options. Because of the above mentioned known carcinogenic pathways, the identification of new targeted therapies, directed to molecular changes within tumor cells, may offers a promising direction towards an improved, more personalized treatment approach.

To be able to apply more personalized treatments, prediction of therapy response and toxicity is needed. In this way, the most appropriate treatment approach for each individual patient can be selected resulting in improved treatment efficacy and reduced side effects. A multitude of prognostic and predictive biomarkers has been suggested, with little success in translating findings to clinical practice until now.[77-79] Therefore, reliable patient-derived tumor models would allow functional analysis of treatment efficacy in a personalized setting. Furthermore, in HPV-positive HNSCC, the development of predictive tumor models and corresponding biomarkers is essential in the context of

treatment de-escalation, which could be considered because of the better prognosis of these tumors.[60,61] However, a subgroup of 10-20% of HPV-positive patients, which cannot be identified until now, show a biological behavior comparable to HPV-negative tumors, resulting in a poor prognosis.[80] In this group, patient-derived tumor models and biomarkers (e.g., HPV genome integration), which are capable to distinguish this therapy insensitive group from tumors with a good prognosis would be of great value for the further development of de-intensified therapy strategies in HPV-positive HNSCC.

Aim and outline of this thesis

The clinical challenges mentioned above necessitate the evaluation of new opportunities to improve treatment and prognosis of HPV-positive and HPV-negative HNSCC. This thesis aims to; 1) assess and increase HPV awareness among the population and general practitioners (GPs) in The Netherlands with the goal to increase HPV vaccination coverage and stimulate early detection; 2) improve the knowledge on HPV genome integration and associated detection techniques in order to identify prognostic biomarkers; 3) investigate potential new targeted therapies to improve treatment options; and 4) explore the suitability and applications of tumor-derived culture models to guide personalized treatment for HNSCC patients.

In **Chapter 2** and **Chapter 3** of this thesis, the knowledge of HPV and the link with OPSCC were assessed in a cross-sectional survey study in the Dutch population and among Dutch GPs, respectively. The results of the study among GPs were summarized in an infographic to be shared with Dutch GPs in **Chapter 4**. In **Chapter 5**, the current literature on HPV genome integration in HNSCC and corresponding detection methods was comprehensively summarized and reviewed. **Chapter 6** describes the development and validation of a novel sequencing-based approach for HPV integration detection in both cell lines and formalin-fixed, paraffin-embedded (FFPE) tissues. In **Chapter 7** and **Chapter 8** the *in-vitro* efficacy of the antiviral agent cidofovir as well as CDK4/6 and PI3K/Akt/mTOR pathway inhibitors was assessed. In **Chapter 9**, reported tumor-derived culture models for HNSCC are compared with each other and evaluated for their suitability as a preclinical prediction model. In **Chapter 10**, an application for the histoculture model for ex-vivo assessment of radiosensitivity is described. In the general discussion of **Chapter 11**, the findings of this thesis are discussed and reflected upon. Finally, a summary of the findings in this thesis is provided.

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PART I

HPV awareness and viral genome integration

Chapter 2

Public awareness of the association between human papillomavirus and oropharyngeal cancer in The Netherlands

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Abstract

Background

Early diagnosis of human papillomavirus (HPV) associated oropharyngeal cancer (OPC) is associated with improved survival. To achieve early diagnosis, it might be beneficial to increase awareness of the link between HPV and OPC. This increase of awareness could also be an important way to increase vaccination rates. The aim of our study was to explore the current public knowledge in the Netherlands regarding the association of HPV with OPC.

Methods

An online cross-sectional survey was used and sent by the company Flycatcher Internet Research to 1539 of their panel members. Data were analyzed statistically by gender, age, educational level and the participants' use of alcohol and tobacco.

Results

The response rate was 68% (1044 participants). Our data revealed that 30.6% of the participants had heard of HPV. There was a knowledge gap regarding HPV in males ($p<0.001$), people older than 65 years ($p<0.001$), people with low education level ($p<0.001$) and current smokers ($p<0.001$). Of the respondents who had heard of HPV, only 29.2% knew of the association between HPV and OPC. We also found that only 49.7% of the population knew of the existence of an HPV vaccine.

Conclusions

The results of this survey indicate that the public awareness of HPV and the association of HPV with OPC is lacking. Interventions to increase awareness of HPV and its association with non-cervical cancer should be considered. This might help to increase the HPV vaccine uptake both for girls and boys and earlier diagnosis of this disease leading to improved survival.

Introduction

Head and neck squamous cell carcinoma (HNSCC) has been the seventh most common cancer worldwide in 2018, accounting for 3% of all cancers.[1] The majority of HNSCC cases are tobacco and alcohol associated, but research in the past decades has highlighted the increasing importance of human papillomavirus (HPV) infection as a risk factor for developing HNSCC, especially for oropharyngeal carcinomas (OPC).[2] While the incidence of tobacco related disease has declined in the past two decades, there is an increase in HPV associated OPC.[2,3] The HPV associated oropharyngeal tumors have different properties than the HPV negative HNSCC; patients are younger, more often male and non-smokers and non-drinkers. In addition, HPV associated OPC is more often seen in the population with a higher socio-economic class.[4] Individuals with frequent oral sex encounters, a greater number of different sexual partners, and earlier sexual experiences seem to be at a higher risk for HPV associated OPC development.[5-7] Earlier diagnosis of HPV associated OPC is associated with improved survival.[8] To achieve early diagnosis, it might be beneficial to increase awareness of the link between HPV and OPC.

Recent data from the United States suggest that the incidence of HPV related OPC exceeds the incidence of HPV related cervical cancer in high income countries, although some reservations must be made because of regional differences.[9,10] The HPV vaccine not only protects against the development of cervical cancer, but also against oropharyngeal cancer.[11] In the Netherlands, since 2009 girls aged 13 years have been offered an HPV vaccination to prevent cervical cancer development from the National Vaccination Program.[12] The vaccine has been included in the vaccination program for boys since the beginning of 2021. Children will also be vaccinated at a younger age from 2021, namely from the age of 9. To maximize the potential benefits of HPV vaccination, it is necessary to get the vaccination coverage as high as possible. The national vaccination coverage for HPV for girls was 53% in The Netherlands in 2019.[13] Because the parents decide on the vaccination, it is important that they are aware of the association between HPV and not only cervical cancer, but also OPC.

Since vaccination against HPV became available, awareness of HPV has dramatically increased.[14] A study by Williams *et al.* under the general public in the United States showed that most respondents were aware that HPV is a causative agent of cervical cancer. However, the majority were not aware of the association between HPV and oropharyngeal cancer.[15] Data from a recent study regarding the public awareness of HPV associated oropharyngeal cancer in men and women in the United Kingdom, showed

that 37% of the respondents had ever heard of HPV and of these 38.7% recognized HPV as a risk factor for OPC.[16]

The aim of our study was to explore the current public knowledge in The Netherlands regarding the association of HPV with oropharyngeal cancer. Our findings will help us to determine if there is need to increase public education on HPV and oropharyngeal cancer. By increasing education and uptake of the HPV vaccine, we hope to combat the development of HPV associated oropharyngeal cancers and other HPV associated tumors.

Methods

Survey design and administration

The medical ethics review committee of Maastricht University Medical Centre approval was obtained on the basis that data collection was anonymized and no vulnerable participants were involved.

A short questionnaire was already developed by Lechner *et al.* (see[16] and[17]), which was kindly provided to us and which we have adapted to our situation. The questionnaire of nine items (see Supplementary data) assessed the knowledge of HPV, of OPC risk factors and symptoms, of the association between HPV and OPC, the knowledge of the HPV vaccine and the participants use of alcohol and tobacco. Tobacco use was divided into current smoker, former smoker and, non-smoker (never smoked), and alcohol consumption was classified in 1-7 drinks per week, 8-14 drinks per week, 15-21 drinks per week, more than 21 drinks per week or no drinks. Demographic characteristics of the participants were provided to us by the company Flycatcher Internet Research, as they sent the online questionnaire to their panel members. These characteristics included gender, age, education level, and living in which province. Education level was measured as low, middle and high. Low was defined as having no certificate or having a certificate of pre-vocational secondary education or secondary vocational education. Middle was defined as having a certificate of intermediate vocational education, or senior general secondary education or pre-university education or having a first year's degree in higher professional education or in university education. High was defined as having a certificate of higher professional education or of university education or having a doctoral or post-doctoral degree.

The company Flycatcher Internet Research sent the online questionnaire to the research group selected from a sample from their panel consisting of people older than 18 years who have registered voluntarily. The sample was stratified by gender, age, educational level, and province. This guarantees that the people in the sample were a representative reflection of the Dutch population aged 18 and older. The selected panellists received an e-mail describing the study, and interested respondents were directed to a website where the survey could be completed. The intended response rate was 1000 participants. Respondents were encouraged to completely fill out the whole survey. Incompletely filled surveys were excluded in the analysis.

Statistical analysis

Statistical analyses were performed using SPSS statistical software for Windows, version 25 (IBM). Descriptive analyses with calculated measures of central tendency and variation were computed, along with frequency tables for categorical variables. Whether distributions of categories are different was tested using Chi-square test. The significance level was set at p=0.05.

Results

Participant characteristics

The online questionnaire was sent to 1539 panel members, of whom 1044 completed the questionnaire (response rate 68%). In 16 other questionnaires, one or more questions were skipped and therefore excluded. This population reflected the Dutch population in terms of gender, age, education level and province. The characteristics of the participants are shown in Table 2.1.

Table 2.1 Characteristics of the participants (N=1044).

Characteristics	N	%
Sex		
male	517	49.0
female	527	51.0
Age		
18-29 years	173	17.0
30-65 years	590	56.0
>65 years	281	27.0
Educational level		
low	293	28.0
middle	463	44.0
high	288	28.0
Smoking		
non-smoker	491	47.0
former smoker	426	41.0
current smoker	127	12.0
Alcohol = drinks per week		
No alcohol use	382	37.0
1-7 drinks	504	48.0
8-14 drinks	110	11.0
15-21 drinks	34	3.0
>21 drinks	14	1.0

Knowledge of HPV

Of the 1044 respondents, 30.6% had ever heard of HPV (Table 2.2). Two times more women were aware of HPV than men (41.6% vs. 19.3% $p<0.001$). Participants aged 18-29 years had most often heard of HPV (44.5%) and participants over 65 years the least (10.7%) ($p<0.001$). Participants with a low educational level had heard of HPV less often than participants with a high education level (12.3% vs. 46.9%) ($p<0.001$). Participants who did not smoke more frequently had heard about HPV than those who smoked or had smoked (38.5% vs. 18.9% and 24.9% $p<0.001$). Of the respondents who already had heard of HPV, 79.9% knew that HPV is transmitted during sex, 72.7% that HPV is transmitted during oral sex, 78.4% that HPV is not rare and only 64.6% knew that HPV does not cause HIV (Table 2.3).

Table 2.2 Knowledge about HPV and oropharyngeal cancer in the Dutch population (N=1044).

Characteristics		Yes. I had heard of HPV before today			Yes. I'm aware of an HPV vaccine			Yes. I'm aware of an HPV vaccine AND I knew of HPV			Yes. I knew of the link between HPV and OPC			Yes. I knew of the link between HPV and OPC AND I knew of HPV		
		N	%	p-value	N	%	p-value	N	%	p-value	N	%	p-value	N	%	p-value
		319	30.6		519	49.7		262	82.1[a]		115	11.0		93	29.2	
Sex																
	Male	100	19.3		202	39.1		75	75.0		47	9.1		34	34.0	
	Female	219	41.6	<0.001	317	60.2	<0.001	187	85.4	0.013	68	12.9	0.049	59	26.9	0.20
Age																
	18-29 years	77	44.5		101	58.4		62	80.5		26	15.0		22	28.6	
	30-65 years	212	35.9		313	53.1		179	84.4		71	12.0		61	28.8	
	> 65 years	30	10.7	<0.001	105	37.4	<0.001	21	70.0	0.008	18	6.4	0.008	10	33.3	0.87
Educational level																
	low	36	12.3		118	40.3		29	80.6		19	6.5		10	27.8	
	middle	148	32.0		219	47.3		115	77.7		51	11.0		42	28.4	
	high	135	46.9	<0.001	182	63.2	<0.001	118	87.4	0.046	45	15.6	0.002	41	30.4	0.92
Smoking																
	current smoker	24	18.9		47	37.0		14	58.3		5	3.9		4	16.7	
	former smoker	106	24.9		202	47.4		86	81.8		39	9.2		31	29.2	
	non-smoker	189	38.5	<0.001	270	55.0	0.004	162	85.7	0.011	71	14.5	0.001	58	30.7	0.36
Alcohol = drinks per week																
	1-7 drinks	171	33.9		263	52.2		140	81.9		60	11.9		51	29.8	
	8-14 drinks	22	20.0		50	45.5		16	72.7		8	7.3		7	31.8	
	15-21 drinks	7	20.6		14	41.2		6	85.7		2	5.9		2	28.6	
	>21 drinks	1	7.1		2	14.3		0	0.0		0	0.0		0	0.0	
	no alcohol use	118	30.9	0.076	190	49.7	0.041	100	84.7	0.24	45	11.8	0.303	33	28.0	0.96

[a] Percentage of participants who were aware of an HPV vaccine and did NOT heard of HPV before today = 34.5% HPV = human papillomavirus OPC = oropharyngeal cancer.

Table 2.3 Knowledge about HPV when already heard of HPV.

	Yes		No		Not sure	
	N	%	N	%	N	%
Is HPV rare?	20	6.3	250	78.4	49	15.4
Is HPV transmitted during sex?	255	79.9	29	9.1	35	11.0
Is HPV transmitted during oral sex?	232	72.7	30	9.4	57	17.9
Can HPV cause HIV (Aids)?	22	6.9	206	64.6	91	28.5

HPV = human papillomavirus; HIV = human immunodeficiency virus.

Knowledge about HPV vaccine

Despite knowledge of HPV in 30.6% (n=319) of all participants (mentioned above), we found that 49.7% (n=519) of all participants knew that there is an HPV vaccine available. This is remarkable, because this means that a part of the participants who had no knowledge of HPV knew that there is a vaccine (Table 2.2). Participants older than 65 years were less aware of HPV vaccination (70%, p=0.008), but there was less spread in the knowledge of the HPV vaccine between the different education levels. Current smokers and participants drinking more than 21 alcoholic drinks per week were also less aware of the existence of an HPV vaccine (58.3% and 0% respectively), although the latter group was small (14 persons).

Knowledge about oropharyngeal cancer

In the overall population, 11% knew of the association between HPV and OPC. Interestingly, of the respondents who had heard of HPV, only 29.2% recognized HPV as risk factor of OPC (Table 2.2). In comparison to the knowledge of the existence of HPV, men were now more aware of this link than women (34.0% versus 26.9% p=0.20), but the knowledge of the link was more equal across the different age categories and education levels. Because parents decide whether or not their children will undergo HPV vaccination, we also looked specifically at the participants aged 30-45 years for the knowledge about HPV and OPC. This knowledge was not different from the participants aged 45-65 years (data not shown). Current smokers and participants drinking more than 21 alcoholic drinks per week were again less aware of the link between HPV and OPC (16.7% and 0% respectively).

Participants were confronted with 11 factors and asked whether these were risk factors for OPC or not. Only 26.9% of the participants correctly identified HPV as a risk factor for OPC (Table 2.4), which is higher than the initial 11.0% (mentioned above). Awareness of other well-established risk factors was much higher: for example, smoking (97.3%) and chewing tobacco (74.5%). Excessive alcohol consumption, poor oral hygiene and

chewing of betel leaf, catchu and areca nuts were less recognized (60%, 38.1% and 30.4% respectively).

Before this question, the participants were asked with an open question what they think could affect a person's chance of throat cancer. Notable factors mentioned include poor air quality (94 times), harmful chemicals (84 times), hot drinking (42 times) and spicy food (17 times).

Table 2.4 Knowledge of reported risk factors for oropharyngeal cancer in the general Dutch population (N=1044).

Risk factor	Yes		No		Not sure	
	N	%	N	%	N	%
Excessive alcohol consumption	626	60.0	139	13.3	279	26.7
Smoking	1016	97.3	10	1.0	18	1.7
Chewing of tobacco	778	74.5	48	4.6	218	20.9
Chewing of betel leaf, catchu and areca nuts	317	30.4	87	8.3	640	61.3
Marijuana use	547	52.4	109	10.4	388	37.2
Poor oral hygiene	398	38.1	274	26.2	372	25.6
Herpes simplex virus infection	277	26.5	139	13.3	628	60.2
Human papilloma virus infection	281	26.9	112	10.7	651	62.4
Family history of cancer	646	61.9	136	13.0	262	25.1
Low fruit and vegetable consumption	253	24.2	338	32.4	453	43.4
Sun exposure	167	16.0	454	43.5	423	40.5

Discussion

Over the past three decades, there has been a clear decrease in the prevalence of tobacco use and an associated decline in tobacco related head and neck cancers in many industrialized countries. The incidence of HPV positive OPC, however, is increasing worldwide, predominantly among men.[2,3] Recent data in the United States suggest that the incidence of HPV related OPC exceeds the incidence of HPV related cervical cancer in high income countries, although some reservations must be made because of regional differences.[9] The HPV vaccine not only protects against cervical cancer, but also against oropharyngeal cancer.[11] Several studies suggested that the public is relatively well informed about HPV as a sexually transmitted disease and of the relationship between HPV and cervical cancer.[14] In contrast, there seems to be a lack of knowledge about the association of HPV and OPC.[15,16]

The present study focused on the awareness of the Dutch population concerning the association between HPV and OPC. Our data revealed that 30.6% of the population had heard of HPV and that this knowledge was less in males, people older than 65 years, low

education level and current smokers. Of the respondents who had heard of HPV, only 29.2% knew of the association between HPV and OPC. This frequency is slightly lower in comparison with earlier studies, for example the study of Williams *et al*., in which 36% of the respondents reported to know that HPV is a causative factor for OPC.[15] An explanation could be the fact that more than 75% of the participants in the study of Williams were aged between 18 and 35, while in our study only 17% of the respondents were aged 18-29 years and 56% aged 30-65 years. In the study of Lechner *et al*. however, 38.7% of the respondents knew of the association between HPV and OPC[16] and the age range of the participants was comparable with that in our study. The participants of our study who were aware of HPV were in general well aware of the prevalence and the (oral) sexually transmission of HPV.

We also found that 49.7% of the population knew of the existence of an HPV vaccine, this percentage was remarkable because it is higher than the percentage of the population knowing of the virus itself. So, 34.5% of the respondents who had never heard of HPV, were aware of the presence of an HPV vaccine. One explanation for this difference could be that the addition of 'vaccine' to 'HPV' increases the knowledge because it creates an association, which people have less with the word 'HPV' alone. Another explanation could be that people don't know what the HPV vaccine is for. In addition, it was striking that if we asked in an open question whether the participants knew about the link between HPV and OPC, only 11% answered positively, whereas when we presented the respondents a list of causative factors for OPC, 26.9% indicated that there was an association between HPV and OPC. We think this is because of the respectively closed versus open way of asking the question.

The greater awareness among women about HPV, the HPV vaccine, and the link of HPV with OPC, suggest that this knowledge is primarily due to awareness of the role of HPV in uterine cervical cancer. Since the incidence of HPV related OPC is 3 to 6 times higher in men than in women and the HPV related OPC exceeds the incidence of HPV related cervical cancer in the higher income countries[3,18], greater awareness of the role of HPV infection in OPC is necessary to improve vaccine uptake, in women but especially also in men.

The knowledge about the association between HPV and OPC was highest in the group with a higher education level and among non-smokers and non-drinkers. This is beneficial because this group has the highest risk of getting HPV associated OPC. However, in general, the knowledge is still substantially low so that more awareness is needed. In addition, greater awareness of the disease may prompt patients harbouring

symptoms of HPV positive cancers to go to the physician in time. Subsequently, the physician must be sufficiently aware of symptoms and risk factors of OPC. A recent study by Lechner *et al*. reported that the level of awareness of HPV and OPC among general practitioners was high, however, the characteristics of HPV associated OPC were less well recognized, indicating the need for further education.[17] Therefore, studying the awareness of HPV and OPC, other risk factors and symptoms among the general practitioners in the Netherlands should also be considered.

There are some limitations of this study that should be considered when interpreting its results. All Internet-based surveys incur the potential for bias by excluding participants who lack Internet connections.[19] Moreover, in this particular study there is also the potential for bias because of the selection of people who want to participate in a panel. Internet surveys are also vulnerable for bias due to nonresponse. As a consequence, the participants may differ significantly from the general population.[20] However, the results of this survey are largely consistent with previously published data on HPV awareness.[15,16,21] This survey was conducted during the COVID pandemic, which may result in an increased interest in virus vaccines and could therefore have influenced the response rate.

In conclusion, the results of this survey indicate that the public awareness of HPV and the association of HPV with oropharyngeal cancer is lacking. Interventions to increase awareness of HPV and its association with non-cervical cancer should be considered. This might help to increase the HPV vaccination uptake and earlier diagnosis of this disease leading to improved survival.

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Supplementary data

Questionnaire about throat cancer

1. What things do you think affect a person's chance of throat cancer? If you cannot think of any, please "don't know" in the box below.

2. Which of the following are common factors for an increased risk of getting throat cancer?

	Yes	No	Not sure
Excessive alcohol consumption	o	o	o
Smoking	o	o	o
Chewing of tobacco	o	o	o
Chewing of Betel leaf/ Catchu and areca nuts	o	o	o
Marijuana use	o	o	o
Poor oral hygiene	o	o	o
Herpes simplex virus infection	o	o	o
Human papillomavirus infection	o	o	o
Family history of cancer	o	o	o
Fruit and vegetable consumption	o	o	o
Sun exposure	o	o	o

3. There are many warning signs and symptoms of throat cancer. Please name as many as you can. If you cannot think of any, please type "don't know" in the box below.

4. Before today had you ever heard of HPV (human papillomavirus)?
   - o Yes
   - o No
   - o Not sure

HPV is the virus that causes cervical cancer.

5. Please read the following statements and say whether you think each one is true or false

	True	False	Not sure
HPV is very rare	o	o	o
HPV can be passed on during sex	o	o	o
HPV can be passed on during oral sex	o	o	o
HPV can cause HIV/AIDS	o	o	o
There is a vaccine against the virus HPV	o	o	o

6. Were you aware that the virus HPV (human papillomavirus) is a risk factor for throat cancer?
   - o Yes
   - o No

Finally, a number of background questions follow below.

7. Do you smoke?
   - o Yes, I'm a current smoker
   - o Yes, I have smoked in the past
   - o No

8. How many cigarettes do you smoke per day?
   - o Less than 10 per day
   - o 10 - 19 per day
   - o 20 - 35 per day
   - o 35 of more per day

9. How many units of alcohol do you consume in the average week?
   - o 1-7
   - o 8-14
   - o 15-21
   - o More than 21
   - o I never drink alcohol

Chapter 3

Awareness of HPV-associated oropharyngeal cancers among GPs in The Netherlands: a cross-sectional study

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Abstract

Background

The incidence of human papillomavirus (HPV)-associated oropharyngeal cancer (OPC) is increasing in high-income countries. HPV-associated OPC generally presents as an invasive disease, often with lymph node involvement, in relatively young patients with minimal or no history of smoking and alcohol consumption. Knowledge on HPV-associated OPC among primary care professionals is essential for disease recognition and early start of treatment.

Aim

To examine the knowledge on HPV-associated OPC among general practitioners (GPs) in The Netherlands.

Design and setting

Cross-sectional postal survey among GPs in The Netherlands.

Method

A twelve-item questionnaire was sent to 900 randomly selected general practices. Outcome measures included awareness of the link between HPV and OPC, epidemiological trends and patient characteristics. Data were statistically analyzed for gender, years after graduation, and self-rated knowledge of OPC.

Results

207 GPs participated in this study. 72% recognized HPV as a risk factor for OPC and 76.3% was aware of the increasing incidence rate of HPV-associated OPC. In contrast, 35.3% of participants knew that HPV-associated OPC patients are more often male, and just over half (53.6%) of the participants were aware of the younger age of these patients.

Conclusion

More than a quarter of GPs in The Netherlands is unaware of HPV as a causative factor for OPC. Furthermore, there is a gap in knowledge on HPV-associated OPC patient characteristics. Further training on these topics could improve disease recognition and ultimately patient survival.

Introduction

Head and neck cancer (HNC) has been the seventh most common cancer worldwide in 2018, accounting for 3% of all cancers.[1] Five-year, age-standardized, relative survival rates range from 25% to 60%, depending on anatomical location, human papillomavirus (HPV) status, and stage at diagnosis.[2] HNC is usually diagnosed in elderly patients in association with tobacco use and heavy alcohol consumption.[3-5] In addition, infection with high-risk HPV, primarily HPV type 16, has been recognized as a major risk factor for the development of HNC, specifically oropharyngeal cancer (OPC). Partly as a result of the worldwide decline in tobacco use, the incidence of HNC incidence has decreased over recent decades. Conversely, the incidence of HPV-associated OPC is increasing in so-called high-income countries, including Australia, the United States, Canada, Sweden, Denmark, and The Netherlands.[3,6-9] A meta-analysis including 5,396 OPCs observed an increase in the proportion of HPV-related OPC from 40.5% before 2000 to 72.2% after 2005, with significant increases in North America and Europe.[10] In the Netherlands, an increase in the prevalence of HPV in OPC was observed from 5.1% in 1990 to 29% in 2010.[9] More recent studies showed a prevalence of HPV in 30-50% of the OPC cases in The Netherlands.[11-13]

HPV-associated OPC is considered to be a distinct clinical and molecular entity.[14,15] In contrast to patients with non-HPV-associated OPC, patients with HPV-associated OPC are younger, more often male, have a higher socioeconomic status and more lifelong sexual partners, and are less likely to have a history of extensive tobacco and alcohol use.[3,15,16] Compared to non-HPV-associated tumors, HPV-associated tumors are generally characterized by a better prognosis, primarily because they are more responsive to chemotherapy and radiotherapy.[17,18] Despite this beneficial treatment response, HPV-associated tumors often have a peculiar clinical presentation. Compared to non-HPV-associated tumors, HPV-associated tumors generally present as smaller (asymptomatic) tumors, but often with regional lymph node metastases and sometimes even with presentation of neck metastases from an occult primary tumor.[19-21] Diagnosis of oropharyngeal HPV-associated tumors at earlier disease stage is associated with improved overall -and disease-specific survival rates.[22] Furthermore, HPV-associated OPC precursor lesions are scarce, unlike cervical cancer, which makes that no validated preventative screening method has been developed for these tumors.[23-25] Therefore, early disease recognition by primary care professionals and no delay in treatment is crucial for patient outcomes.

Recognizing patients at risk for HPV-associated OPC can pose challenges for general practitioners (GPs), who may not have detailed knowledge of the disease and

corresponding patient characteristics. A systematic review by Dodd *et al.* identified 41 studies investigating the knowledge about the link between HPV and OPC in different populations.[26] This study revealed that the lowest knowledge was observed in the general population (1-44%), which we could confirm in a recent study in The Netherlands showing that only 11% of the general population was aware of the link between HPV and OPC (29.2% of people that stated to be aware of the existence of HPV).[27] The same systematic review reported that the highest knowledge on HPV in OPC was reported among medical and dental professionals (26-91%), which was also found by a recent study by Lechner *et al.* in the UK, reporting that 74% of GPs recognized HPV as a risk factor for OPC.[28]

This study is the first to assess awareness of the link between HPV and OPC, the epidemiological trends in (HPV-associated) OPC and demographic profiles of patients with HPV-associated OPC among a randomly selected group of GPs in The Netherlands. The results might identify areas where further education for GPs is needed to increase specific knowledge and thereby improve disease recognition and patient outcomes.

Methods

Survey design

We performed a cross-sectional questionnaire survey among GPs in The Netherlands. A short questionnaire was adapted and translated from an already developed questionnaire by Lechner *et al.*[28] (Supplementary File S3.1). This questionnaire assessed demographic characteristics of participants, self-rated knowledge of OPC, awareness of OPC risk factors, knowledge on the association between HPV and OPC, and characteristics of patients with HPV-associated OPC. Demographic characteristics included gender, years since graduation, and current position. Self-rated knowledge on OPC was assessed by a Likert scale. To assess the awareness of risk factors, eleven risk factors (of which eight correct and three false) were selected from epidemiological literature. The medical ethical committee of Maastricht University Medical Center gave approval for data collection, on a basis that data were anonymized, and no vulnerable participants were involved (METC 2020-1887).

Participants

The postal addresses of 900 GPs throughout The Netherlands were obtained from The Netherlands Institute for Health Services Research (NIVEL). These 900 GPs were selected

by random sampling of all GPs registered at NIVEL, comprising approximately 85%-90% of all GPs in The Netherlands. A response rate of 20% was anticipated based on previous surveys among GPs (NIVEL, institutional communication). The questionnaire was administered in September 2020 to the GPs by mail. To increase the response rate, questionnaires could be completed both in paper format and by a link to the online platform Survey Monkey. In addition, a reminder was sent two weeks after the initial invitation. Answers of returned paper questionnaires were added as separate collectors to the Survey Monkey database. Both paper format and online questionnaires were collected anonymously. After completing the questionnaire, participants were given a factsheet with information about HPV and HPV-associated OPC.

Statistical analysis

Statistical analyses were performed using SPSS statistical software for Windows, version 20 (IBM), and Stata version 14.1. Descriptive analyses with calculated measures of central tendency and variation were computed, along with frequency tables for categorical variables. Whether distributions of categories are different was tested using Chi-square tests and Likelihood Ratio tests. The extended Mantel-Haenszel Stratified Test of Association was used to test for linear trends. For this, variables were recoded into two categories (the 'correct' answers and 'incorrect answers'). P-values below 0.05 were considered statistically significant.

Results

Participant's characteristics

The questionnaire was sent to 900 GPs throughout The Netherlands. Overall, 212 questionnaires were collected, resulting in a response rate of 23.6%. The majority of the questionnaires was completed in paper format compared to the online questionnaire (141 vs. 71). Five questionnaires were incomplete (6 to 9 missing answers of 12 questions in total) and therefore excluded from analysis. The demographic characteristics of participants are shown in Table 3.1. Owing to the applied privacy legislation, it was not possible to compare features between responders and non-responders. Nevertheless, responders could be compared to the whole registry of GPs in The Netherlands (in 2019) for sex, current position, and GP experience.[29,30] Supplementary Table S3.1 shows that only the percentage of female GPs is different between the whole registry (58%) versus the present study population (48%). Notably, 49 out of 207 responding GPs (23.7%) rated their knowledge of OPC as 'poor'.

Table 3.1 Demographic characteristics and self-rated knowledge of OPC of 207 participating GPs in The Netherlands (2020).

Characteristics	N	%
Stage of training/position		
GPST year 1	2	1
GPST year 2	0	0
GPST year 3	7	3.4
GP	198	95.7
Sex		
Male	107	51.7
Female	100	48.3
Years since graduation		
Still in training	9	4.3
<2 years	7	3.4
2-5 years	18	8.7
5-10 years	39	18.8
10-20 years	59	28.5
>20 years	75	36.2
Self-rated knowledge of OPC		
Poor	49	23.7
Sufficient	148	71.5
Good	10	4,8
Very good	0	0

GPST = General Practitioner Specialty Training; OPC = Oropharyngeal cancer.

Knowledge of HPV and risk factors for OPC

Of all 207 responders, 72% was aware of the link between HPV infection and OPC, whereas 23.7% was not aware of this link and 4.3% was not sure (Table 3.2). To assess awareness of risk factors for OPC in general, respondents were confronted with eleven risk factors and asked whether these present risk factors for OPC or not (Table 3.3). Infection with HPV was recognized as a risk factor for OPC by 78.7% of participants. Participants showed to have good knowledge of the risk factors smoking, alcohol abuse and chewing of tobacco (100%, 98%, and 91.3%, respectively). Chewing of betel leaf/betel palm/betel nut (Areca nut), poor oral hygiene, family history, and low fruit and vegetable consumption were less well recognized as risk factors (28.0%, 51.7%, 56.5%, and 31.4%, respectively).

Over three-quarters of participants was aware of the increase of HPV-associated OPC cases over the past two decades (76.3%). A linear trend with years after graduation was not observed (p=0.265). In contrast, only 19.8% was aware of the decrease in smoking associated OPC rates during the same period. Interestingly, male GPs were significantly more aware of this decrease compared to female GPs (p=0.021) (Table 3.2).

Table 3.2 Knowledge of HPV as risk factor for OPC and epidemiological trends of OPC incidence among 207 GPs in The Netherlands (2020)

		Total (%)	Sex (%)			Years after graduation as GP (%)						Self-rated knowledge of OPC (%)			
			Female	Male	*p*-value	<2[a]	2-5	5-10	10-20	>20	*p*-value	Poor	Sufficient	Good	*p*-value
Were you aware of the link between HPV and OPC before today?	Yes	149 (72.0%)	80 (74.8%)	69 (69.0%)	0.273	14 (87.5%)	14 (77.8%)	31 (79.5%)	39 (66.1%)	51 (68.0%)	0.267	29 (59.2%)	112 (75.7%)	8 (80.0%)	0.216
	No	49 (23.7%)	21 (19.6%)	28 (28.0%)		2 (12.5%)	2 (11.1%)	7 (17.9%)	16 (27.1%)	22 (29.3)		17 (34.7%)	30 (20.3%)	2 (20.0%)	
	Not sure	9 (4.3%)	6 (5.6%)	3 (3.0%)		0 (0.0%)	2 (11.1%)	1 (2.6%)	4 (6.8%)	2 (2.7%)		3 (6.1%)	6 (4.1%)	0 (0.0%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	
Over the past two decades, HPV associated OPC rates have:	Increased	158 (76.3%)	80 (74.8%)	78 (78.0%)	0.135	10 (62.5%)	11 (61.1%)	35 (89.7%)	42 (71.2%)	60 (80.0%)	0.020[b]	36 (73.5%)	114 (77.0%)	8 (80.0%)	0.664
	Decreased	6 (2.9%)	2 (1.9%)	4 (4.0%)		2 (12.5%)	2 (11.1%)	0 (0.0%)	0 (0.0%)	2 (2.7%)		1 (2.0%)	5 (3.4%)	0 (0.0%)	
	Stayed the same	8 (3.9%)	7 (6.5%)	1 (1.0%)		2 (12.5%)	1 (5.6%)	2 (5.1%)	2 (3.4%)	1 (1.3%)		4 (8.2%)	4 (2.7%)	0 (0.0%)	
	Not sure	35 (16.9%)	18 (16.8%)	17 (17.0%)		2 (12.5%)	4 (22.2%)	2 (5.1%)	15 (25.4%)	12 (16.0%)		8 (16.3%)	25 (16.9%)	2 (20.0%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	
Over the past two decades. smoking associated OPC rates have:	Increased	96 (46.4%)	58 (54.2%)	38 (38.0%)	0.021	7 (43.8%)	10 (55.6%)	19 (48.7%)	26 (44.1%)	34 (45.3%)	0.354	26 (53.1%)	64 (43.2%)	6 (60.0%)	0.219
	Decreased	41(19.8%)	15 (14%)	26 (26.0%)		4 (25.0%)	4 (22.2%)	8 (20.5%)	13 (22.0%)	12 (16.0%)		5 (10.2%)	34 (23.0%)	2 (20.0%)	
	Stayed the same	42 (20.3%)	17 (15.9%)	25 (25.0%)		4 (25.0%)	4 (22.2%)	4 (10.3%)	10 (16.9%)	20 (26.7%)		9 (18.4%)	31 (20.9%)	2 (20.0%)	
	Not sure	28 (13.5%)	17 (15.9%)	11 (11.0%)		1 (6.3%)	0 (0.0%)	8 (20.5%)	10 (16.9%)	9 (12.0%)		9 (18.4%)	19 (12.8%)	0 (0.0%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	

OPC = Oropharyngeal cancer; HPV = Human papillomavirus; *p*-values were calculated with Chi-square tests or likelihood ratio tests; [a] = also includes GPs still in training; [b] = no statistically significant trend observed with the Extended Mantel-Haenszel test.

Table 3.3 Knowledge of reported risk factors for OPC among 207 GPs in The Netherlands (2020).

	Yes		No		Not sure	
Risk factor	N	%	N	%	N	%
Smoking	207	100.0	0	0.0	0	0.0
Alcohol abuse	203	98.1	1	0.5	3	1.4
Chewing of tobacco	189	91.3	4	1.9	14	6.8
Chewing of betel leaf/palm/nut	58	28.0	12	5.8	137	66.2
Marijuana use	106	51.2	24	11.6	77	37.2
Poor oral hygiene	107	51.7	54	26.1	46	22.2
Herpes simplex virus infection	27	13.0	99	47.8	81	39.1
Human papilloma virus infection	163	78.7	9	4.3	35	16.9
Positive family history	117	56.5	40	19.3	50	24.2
Low fruit and vegetable consumption	65	31.4	47	22.7	95	45.9
Sun exposure	34	16.4	110	53.1	63	30.4

Knowledge of HPV-associated OPC patient characteristics

Knowledge on HPV associated OPC patient characteristics among GPs is essential for disease recognition and early start of treatment. Only 35.7% of all participants knew that OPC patients with HPV-associated tumors are more often male, whereas a comparable percentage (34.3%) was not sure (Table 3.4). GPs who rated their knowledge of OPC as 'good' were more aware of this gender difference (p=0.003). However, this is a small group of only 10 GPs (4.8% of total, Table 3.1) and a linear trend for self-rated knowledge of OPC and awareness of the male gender of patients was not observed (p=0.152).

That HPV-associated OPC patients are generally younger than 60 years of age was correctly recognized by just over half of participants (53.6%). Interestingly, GPs with a self-rated knowledge of 'good' were less well aware of the younger age of these patients, but no statistically significant trend was observed (p=0.981). Markedly, only 17.4% was aware that HPV-associated OPC patients generally have a better prognosis compared to non-HPV-associated OPC patients. Despite the small group size, GPs still in training or graduated less than 2 years ago were more aware of this better prognosis (33.3% for GPs in training and 42.9% for <2 years after graduation) compared to their colleagues who graduated more than 2 years ago (16.7%, 15.4%, 23.7%, and 9.3% for 2-5, 5-10, 10-20, and >20 years after graduation, respectively). A trend towards significancy was observed (p=0.054). More than half of all GPs were not sure about prognosis of these patients (57%) (Table 3.4).

Table 3.4 Knowledge of HPV-associated OPC patient characteristics and prognosis among 207 GPs in The Netherlands (2020).

		Total (%)	Sex (%)			Years after graduation as GP (%)						Self-rated knowledge of OPC (%)			
			Female	Male	*p*-value	<2[a]	2-5	5-10	10-20	>20	*p*-value	Poor	Sufficient	Good	*p*-value
OPC patients with HPV associated tumors are more often:	Male	74 (35.7%)	38 (35.5%)	36 (36.0%)	0.415	6 (37.5%)	4 (22.2%)	17 (43.6%)	21 (35.6%)	26 (34.7%)	0.424	16 (32.7%)	51 (34.5%)	7 (70.0%)	0.003[b]
	Female	35 (16.9%)	14 (13.1)	21 (21.0%)		4 (25.0%)	4 (22.2%)	5 (12.8%)	11 (18.6%)	11 (14.7%)		3 (6.1%)	31 (20.9%)	1 (10.0%)	
	Equal	27 (13.0%)	16 (15%)	11 (11.0%)		1 (6.3%)	1 (5.6%)	8 (20.5%)	10 (16.9%)	7 (9.3%)		4 (8.2%)	23 (15.5%)	0 (0.0%)	
	Don't know	71 (34.3%)	39 (36.4)	32 (32.0%)		5 (31.3%)	9 (50.0%)	9 (23.1%)	17 (28.8%)	31 (41.3%)		26 (53.1%)	43 (29.1%)	2 (20.0%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	
OPC patients with HPV associated tumors are more often:	Age <60 years	111 (53.6%)	54 (50.5%)	57 (57%)	0.325	9 (56.3%)	10 (55.6%)	24 (61.5%)	30 (50.8%)	38 (50.7%)	0.871	23 (46.9%)	86 (58.1%)	2 (20.0%)	0.018[b]
	Age >60 years	42 (20.3%)	26 (24.3%)	16 (16%)		4 (25.0%)	4 (22.2%)	8 (20.5%)	13 (22%)	13 (17.3%)		8 (16.3%)	28 (18.9%)	6 (60.0%)	
	Don't know	54 (26.1%)	27 (25.2%)	27 (27%)		3 (18.8%)	4 (22.2%)	7 (17.9%)	16 (27.1%)	24 (32%)		18 (36.7%)	34 (23.0%)	2 (20.0%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	
The prognosis of patients with HPV positive OPC is generally compared to HPV negative OPC	Better	36 (17.4%)	18 (16.8%)	18 (18%)	0.292	6 (37.5%)	3 (16.7%)	6 (15.4%)	14 (23.7%)	7 (9.3%)	0.011[b]	9 (18.4%)	27 (18.2%)	0 (0.0%)	0.157
	Worse	43 (20.8%)	17 (15.9%)	26 (26%)		2 (12.5%)	4 (22.2%)	3 (7.7%)	16 (27.1%)	18 (24%)		6 (12.2%)	35 (23.6%)	2 (20%)	
	Equal	10 (4.8%)	6 (5.6%)	4 (4%)		0 (0.0%)	2 (11.1%)	0 (0.0%)	2 (3.4%)	6 (8.0%)		1 (2%)	8 (5.4%)	1 (10%)	
	Don't know	118 (57%)	66 (61.7)	52 (52%)		8 (50.0%)	9 (50%)	30 (76.9%)	27 (45.8%)	44 (58.7%)		33(67.3%)	78 (52.7%)	7 (70%)	
	Total	207 (100%)	107 (100%)	100 (100%)		16 (100%)	18 (100%)	39 (100%)	59 (100%)	75 (100%)		49 (100%)	148 (100%)	10 (100%)	

GP = General practitioner; OPC = Oropharyngeal cancer; HPV = Human papillomavirus; *p*-values were calculated with Chi-square tests or likelihood ratio tests; [a] = also includes GPs still in training; [b] = no statistically significant trend observed with the Extended Mantel-Haenszel test.

Discussion

Summary

The incidence of HPV-associated OPC is increasing in high-income countries, including The Netherlands.[3,6,8,10] Although these tumors often present with invasive properties and regional lymph node metastases, their prognosis is usually favorable compared to non-HPV-associated tumors.[21] Early disease recognition by primary care professionals and no delay in the start of treatment is crucial for patient outcomes. The aim of this study was to assess, for the first time, the awareness of the link between HPV and OPC and knowledge of associated patient characteristics in a sample of GPs in The Netherlands. Our results show that of the responding GPs; 1) 72% was aware of the link between HPV and OPC; 2) 76.3% was aware that HPV-associated OPC rates have increased over the past two decades; 3) only 35.7%, 53.6%, and 17.4% was aware of gender, age, and prognosis of HPV-associated OPC patients, respectively.

Strengths and limitations

Participants were selected by random sampling of all GPs registered at NIVEL (Netherlands Institute for Health Services Research), comprising 85-90% of all GPs in The Netherlands, minimizing sampling bias. Furthermore, to minimize response bias, GPs were offered the choice to complete the questionnaire via an online link or on paper. Since the response rate was relatively low, and we have no information on non-responders due to applied privacy legislation, we were not able to test for (non)response bias that may affect the interpretation of the results of our study. However, we observed that the percentage of female GPs in our study sample was lower compared to the whole registry of GPs (Supplementary Table S3.1). Furthermore, participants may have looked at subsequent questions when filling in the paper format questionnaire, which may have influenced their answers. In the online questionnaire, questions could only be answered in sequence. When comparing the online format questionnaires with the paper format questionnaires, however, no difference was observed in awareness of HPV in OPC (73.9% vs. 71.0%, respectively).

Comparison with existing literature

Previous studies investigating the knowledge on the role of HPV in HNC among medical and dental professionals show varying awareness rates from 26-91%.[26] The awareness rate of GPs in this study (72%) is comparable to the awareness reported for GPs in the UK (74%) and Poland (80%).[28,31] The latter study used different outcome variables to assess knowledge of HPV-associated OPC, by asking “How important is the impact of

HPV on the development of upper respiratory tract pathology?", rather than "Have you heard about the link between HPV and OPC before today?" (Table 3.5). This might induce bias in the interpretation of the actual awareness percentage and could make direct comparison difficult. In contrast, the awareness among GPs in our study is higher than in Jordan (43.3%), Germany (54%), and Italy (38%)[32-34] (Table 3.5). However, these studies were performed more than five years ago and increasing knowledge on HPV and OPC over the years and the introduction of the HPV vaccine might have influenced awareness rates among GPs.

Table 3.5 Overview and results of published studies reporting on awareness of HPV in the development of head and neck cancers among GPs and other health care professionals (2014-2018).

Author	Year	Country	Study population	Results	Ref.
Hertrampf	2014	Germany (Schleswig-Holstein)	33 ENTs, 192 GPs, 135 IMs, 28 DERMs	HPV recognized as a risk factor for oral cancer by 70% of ENTs, 54% of GPs, 51% of Internal medicine, and 82% of DERM	[33]
Signorelli	2014	Italy	938 GPs	38% was aware of HPV as a risk factor for oral cancer.	[34]
Jackowska	2015	Poland	144 ENTs, 192 GPs, 68 trainees	Of the GPs, the importance of HPV in the development of OPC was rated as 'Large' by 28.6%, as 'I know the problem' by 44.8%, as 'Overrated' by 6.8%, and as 'Have not heard about the problem' by 19.2%.	[31]
Hassona	2016	Jordan	165 dentists, 165 GPs	43.3% was aware of HPV as a risk factor for oral cancer. No significant difference was found between dentists and GPs	[32]
Lechner	2018	United Kingdom	384 GPs	73.9% was aware of HPV as a risk factor for OPC	[28]

ENT = Ear, nose -and throat physician; GP = General practitioner; IM = Internal medicine physicians; DERM = Dermatologist; HPV = Human papillomavirus; OPC = Oropharyngeal cancer

This study showed that the knowledge on HPV-associated OPC patient characteristics and prognosis is limited. The UK study also noticed this knowledge gap, describing that 41.5% of GPs identified HPV-associated OPC as being more common in men, and 58.8% correctly reported the association with younger age.[28] Interestingly, our results show that GPs in training or recently graduated GPs had greater knowledge of the favorable prognosis. These data suggest that education is necessary to further increase awareness of patient prognosis and demographics of HPV-associated OPC.

Several similar studies among the general population suggest that the awareness of the role of HPV in the development of cervical cancer is relatively high. However, people showed to be less informed about the role of HPV in OPC.[35-37] In a recent study in The Netherlands, we showed that 30.6% of 1,044 participants had heard of HPV and only 29.2% of these (11.0% of all participants) knew about the association between HPV and OPC.[27] Importantly, knowledgeable GPs could play an important role in prevention of HPV-associated disease by educating the general public and encouraging the uptake of the HPV vaccine.

Implications for practice

Our results show that the sample of GPs in this study is reasonably aware of HPV as a causative factor for OPC. Nevertheless, more than a quarter of GPs is still unaware of this link. Particularly, knowledge on less common risk factors and characteristics of patients at risk for HPV-associated OPC should be improved. This knowledge is important as HPV-associated tumors generally present in a relatively young patient population, without typical risk factors, and OPC might therefore be less well recognized in these patients. In the context of educational resources, we have created a factsheet containing information about HPV and OPC, that was sent to all GPs participating in this study. In addition, further training in the form of regional and national meetings might contribute to better targeted knowledge of these topics, leading to HPV-associated disease prevention, improved disease recognition in the primary care setting and ultimately duly referral of patients to secondary care.

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Supplementary data

File S3.1 Questionnaire on HPV and OPC distributed among GPs in The Netherlands (2020).

1. Stage of training/position ☐ GPST year 1 ☐ GPST year 2 ☐ GPST year 3
2. ☐ GP ☐ Other (please state)

3. Gender ☐ Female ☐ Male

4. Years since graduation as GP

   ☐ Still in training ☐ <2 years ☐ 2-5 years ☐ 5-10 years
   ☐ 10-20 years ☐ > 20 years

5. How would you rate your knowledge of oropharyngeal cancers?

   ☐ Poor ☐ Sufficient ☐ Good ☐ Very good

6. There are many warning signs and symptoms of oropharyngeal cancers, please list as many as you can. If you cannot think of any, please write 'don't know' below.

7. Please list risk factors for oropharyngeal cancers. If you cannot think of any, please write 'don't know' below.

8. Which of the following may be risk factors for oropharyngeal cancer?

	Yes	No	Don't know
Smoking			
Alcohol abuse			
Chewing of tobacco			
Chewing of beatel leaf/palm/nut			
Marijuana use			
Poor oral hygiene			
Herpes simplex virus infection			
Human papilloma virus infection			
Positive family history			
Low fruit and vegetable consumption			
Sun exposure			

9. Smoking is one of the risk factors for oropharyngeal cancers. Do you think the rates of smoking-related oropharyngeal cancers in developed countries have changed over the past two decades?

☐ Increased ☐ Decreased ☐ Stayed the same ☐ Don't know

Recently, several discoveries have been made about the association between human papillomavirus (HPV) and oropharyngeal cancers.

10. Before today, had you heard about the link between oropharyngeal cancer and HPV?

☐ Yes ☐ No ☐ Don't know

11. Do you think the rates of human papillomavirus-associated oropharyngeal cancers in developed countries have changed over the past two decades?

☐ Increased ☐ Decreased ☐ Stayed the same ☐ Don't know

12. Patients with HPV-associated oropharyngeal cancers are more often:

    a) ☐ Male ☐ Female ☐ Same gender composition ☐ Don't know

    b) ☐ Younger than 60 years of age ☐ Older than 60 years of age ☐ Don't know

13. The prognosis of patients with HPV-associated oropharyngeal cancer is (fill in) patients with non-HPV-associated oropharyngeal cancer.

    a) ☐ better than ☐ worse than ☐ equal to ☐ Don't know

Thank you for taking the time to complete this questionnaire.

If you would like to receive a document containing background information on HPV and oropharyngeal cancers, please fill in your email address below. We will only use your email address for the purpose of sending you this document, after which it will be deleted.

Email address to receive background information document:

……

Table S3.1 Comparison of demographic characteristics of 207 responding GPs with the whole registry of GPs in The Netherlands in 2019. p-values were calculated using a Chi-square test.

	Study sample of GPs (n=207)	Whole registry of GPs in The Netherlands (n=12,766)	*p-value*
Female GPs	100 (48.3%)	7,405 (58%)	0.0063
Still in GPST	9 (4.4%)	750 (5.8%)	0.6075
GPs graduated >20 years ago / ≥50 years of age	75 (36.2%) *	5,362 (42%) **	0.1100

GP = General practitioner; GPST = General Practitioner Specialty Training; * = number and percentage of GPs graduated > 20 years ago; ** = Number and percentage of GPs ≥ 50 years of age.

Chapter 4

Oropharyngeal cancer caused by the human papillomavirus: an infographic

Imke Demers, Femke Verhees, Leo J. Schouten, Jean W.M. Muris, Bernd Kremer, Ernst-Jan M. Speel

Huisarts Wet 65, 2022;16-17

Oropharyngeal cancer caused by the human papillomavirus

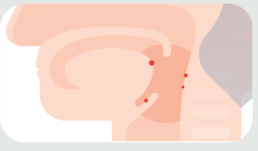
Authors: Imke Demers, Femke Verhees, Leo Schouten, Jean Muris, Bernd Kremer, Ernst-Jan Speel

GENERAL

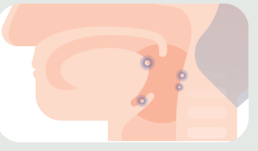
Worldwide, there is an increase in HPV associated oropharyngeal cancer cases. Incidence rates have increased from approximately 5% to 50% over the past 30 years. In Europe, 40-50% of all oropharyngeal cancer cases are associated with HPV infections. In contrast, the incidence rates of HPV negative head and neck cancers are decreasing.

Head and neck cancer is typically associated with the traditional risk factors smoking and excessive alcohol consumption. These risk factors are the cause of approximately 50% of oropharyngeal cancer cases.

HPV positive tumors are more common in (younger) patients without these traditional risk factors, but with a higher soceioeconomic status and higher number of sexual partners.



HPV positive oropharyngeal tumors present, more often than HPV negative tumors, as small (asymptomatic) tumors, not rarely metastasized to the cervical lymph nodes at time of diagnosis.



Nevertheless, the prognosis of HPV positive tumors is generally better, regardless of applied treatment (surgery, chemotherapy and/or radiotherapy.

The sooner the diagnosis and start of treatment, the better the prognosis for the patient.

Sufficient knowledge about HPV positive oropharyngeal cancer and associated patient characteristics will contribute to early recognition of the disease.

The increasing incidence of HPV positive oropharyngeal cancer and the biological and clinical differences with HPV negative tumors highlight the importance of well informed general practitioners (GPs).

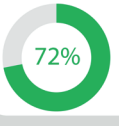
HUISARTS EN WETENSCHAP JULI 2022

RESEARCH

Anually, approximately 3000 new head and neck cancer patients are diagnosed, meaning that a general practitioner (GP) sees a head and neck cancer patient on average once every 4 years. The number of HPV positive oropharyngeal cancer patients is even smaller. Therefore, it is important that medical specialists share their knowledge with GPs. By publishing this study, we aim to increase the knowlegde of GPs about HPV positive oropharyngeal cancer.

Questionnaire survey among
900 general practices in The Netherlands.

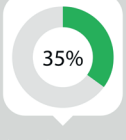
Of all **207 participating** GPs



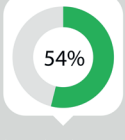
were aware of the link between HPV and the development of oropharyngeal cancer.

76% knew about the increasing incidence of this disease over the past two decades.

Only **20%** were aware of the decrease of HPV negative oropharyngeal cancer cases during the same period.



35% knew that patients with HPV associated oropharyngeal cancer are more often male (75-80%).



54% knew that these patients are often (58%) < 60 years of age at time of diagnosis.

Notably, only **17%** of participating GPs **were aware** of the **better prognosis of HPV associated tumors** compared to HPV negative tumors. The estimated 5-year survival is 80-90% in HPV positive tumors and 50% in HPV negative tumors.

GPs in training or recently graduated GPs were more aware of this favorable prognosis compared to more experienced GPs (33% versus 43%).

In conclusion, one-quarter of Dutch GPs were not aware of HPV as a causative factor for oropharyngeal cancer. In addition, the knowledge about patient characteristics can still be improved. Further training for GPs will contribute to an increased level of knowledge and better recognition of HPV positive oropharyngeal cancer.

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Chapter 5

Causes and consequences of HPV integration in head and neck squamous cell carcinomas: state of the art

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Abstract

A constantly increasing incidence in high-risk Human Papillomavirus (HPV) driven head and neck squamous cell carcinoma (HNSCC), especially of oropharyngeal origin, is being observed. During persistent infections, viral DNA integration into the host genome may occur. Studies are examining if the physical status of the virus (episomal vs. integration) affects carcinogenesis and eventually has further-reaching consequences for disease progression and outcome. Here, we review the literature of the most recent five years focusing on the impact of HPV integration in HNSCC, covering aspects of detection techniques used (from PCR to NGS approaches), integration loci identified, and associations with genomic and clinical data. The consequences of HPV integration in the human genome, including the methylation status and deregulation of genes involved in cell signaling pathways, immune evasion, and response to therapy, are also summarized.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is presently the sixth leading type of cancer worldwide, with 630,000 new patients resulting in over 350,000 deaths annually.[1] Generally, HNSCC originates from the mucosal linings of the upper aerodigestive tract. In more than 90% of the cases, HNSCCs arise in the oral cavity, oropharynx, and larynx[1,2], frequently due to the activation of oncogenes such as epidermal growth factor receptor (*EGFR*), as well as loss-of-function mutations in tumor-suppressor genes such as *TP53* and *CDKN2A*[3]. Treatment of early-stage HNSCC usually comprises surgery and/or radiotherapy. However, for patients with advanced HNSCC, multimodal treatment regimens such as surgery followed by radiation or definitive platinum-based chemoradiation are performed.[2,3] Additionally, in advanced and/or metastasized HNSCC, targeted therapy with the EGFR specific monoclonal antibody cetuximab or immunotherapy using anti-PDL1 antibodies may be incorporated into the patient treatment regime.[2,4-6] Patient treatments unfortunately cause early and late toxicity which severely lower the quality of life.[4] Moreover, preneoplastic sites often persist after treatment, allowing the possibility of local recurrences and second primary tumors which are both responsible for a large proportion of deaths.[2]

HNSCC carcinogenesis can be majorly classified into HNSCC mediated by high-risk human papilloma virus (HPV) infection and HPV-negative HNSCC that is primarily caused by tobacco and alcohol consumption.[7] Over the last decade, a striking increase in HPV-positive HNSCC incidences has been observed in the Western world[2], especially of oropharyngeal squamous cell carcinoma (OPSCC). Up to 90% of the OPSCCs have been associated with HPV.[8] Furthermore, it has been reported that, in the USA, the incidence of HPV-positive HNSCCs has surpassed that of HPV-positive cervical SCCs.[9,10]

Despite the morphological (e.g., poorly differentiated), molecular (e.g., less chromosomal aberrations), and clinical characteristics (e.g., younger age, less tobacco and alcohol consumption) of HPV-positive tumors, patients with this type of HNSCC have a favorable prognosis, regardless of the treatment strategy applied.[2,4,11] This could be attributed to the fact that HPV-positive patients present with fewer genetic alterations, an impaired DNA double strand break repair response, and respond better to radiotherapy due to an intact apoptotic response.[11] The above are likely to be caused by single tumor-initiating events rather than field carcinogenesis. This is generally observed with younger and healthier age groups and hence they display fewer comorbidities. Moreover, radiotherapy and chemotherapy could trigger an immunological response against virus-specific antigens.[12] Nevertheless, additional risk factors such as smoking,

EGFR overexpression, advanced nodal stage, and chromosomal instability can cause poor prognosis in patients with HPV-positive HNSCCs.[8]

For a biologically relevant HPV infection, a couple of events are considered to be essential. Sites of infection involve stratified keratinocyte layers of epidermal origin. The virus particularly prefers functional epithelial appendages, such as salivary glands in the oral cavity and tonsillar crypts, as well as sites where stratified epithelium is adjacent to columnar epithelium, for instance in the uterine cervical transformation zone.[13] These sites are thought to be preferentially targeted because they lack the highly structured barrier function of the epithelium and have an increased occurrence of epithelial reserve cells/stem cells. To hijack these cells, wounds/microlesions are furthermore required to reach the basal cell layer so that it is ensured that actively proliferating cells become infected. At the sites of (micro)injury, an influx of serum containing Heparan sulfate proteoglycan (HSPGs), growth factors (GFs), and cytokines are produced to promote wound healing. Subsequently, HPV L1 capsid protein binds to exposed HSPGs.[14] In addition, virions binding to α6-integrins is required, initiating further intracellular signaling events. In turn, conformational changes induced in HSPGs result in L2 cleavage, binding of the exposed L2 N-terminus to an L2-specific receptor (annexin A2 heterotetramer), and subsequent clathrin-, caveolin-, lipid raft-, flotillin-, cholesterol-, and dynamin-independent endocytosis of HPV16.[15]

Starting from a transient HPV infection, the viral genome maintains as extra-chromosomal episomes. However, persistent infection by high-risk HPVs may lead to the integration of viral genome into the host genome. Viral integration requires both viral and host DNA breakage. Therefore, the rate of integration is expected to be related to the degree of DNA damage, which can be induced by a number of factors (Figure 5.1).[15,16] In particular, excessive amounts of reactive nitrogen and oxygen species originating, for example, from inflammation caused by HPV infection itself (especially through the expression of E6 and E7) or from coinfection with other pathogens, as well as toxic agents originating from environmental or other sources, can cause DNA damage.[17-19] In addition, Apolipoprotein B mRNA-editing catalytic (APOBEC) polypeptides are recently identified as a source of DNA damage, as will be discussed later. Subsequently, there is accumulation of chromosomal alterations and activation of DNA damage repair mechanisms that could promote viral integration. Two possible mechanisms have been proposed by which integration occurs, namely direct insertion and looping integration (Figure 5.2).

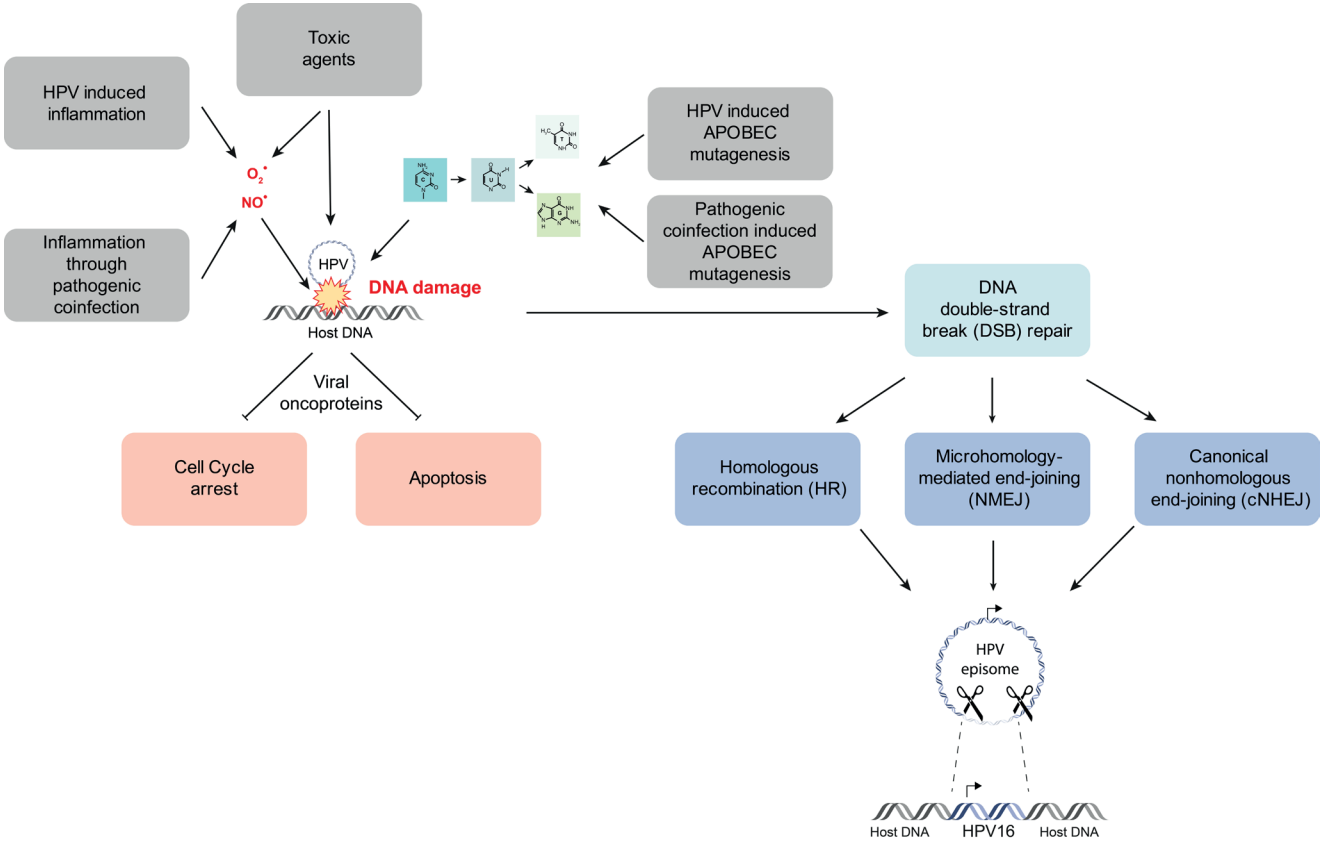

Figure 5.1 Discussed drivers of DNA damage and HPV integration. Intrinsic and extrinsic drivers such as inflammation, toxic agents, or APOBEC mutagenesis caused by HPV infection are able to instigate DNA damage. Subsequently, chromosomal aberrations and DNA damage repair mechanisms might promote viral integration. APOBEC = Apolipoprotein B mRNA-editing catalytic polypeptide.

Direct insertion is thought to occur by a process known as microhomology-mediated end-joining (MMEJ), which can be caused by the interference of HPV oncoproteins with the DNA double-strand break (DSB) repair pathway. MMEJ is highly error-prone and acts as a backup pathway for defects that occur in the homologous recombination (HR) pathways or major canonical non-homologous end-joining (cNHEJ).[20] This can lead to repair events that are lethal. Interestingly, increased microhomology has been observed between HPV virus and viral integration genomic sites in oropharyngeal and cervical cancers, signifying a role of MMEJ. This is achieved when the broken viral genome exploits sequence homology, i.e., identical genomic nucleotide sequence, between the viral ends and the host genome. This is followed by deletion of these microhomologies from both genomes and insertion of the viral genome as a single genome or as concatemerized genomes into the host genome.[21] The DNA looping integration model proposes recurrent patterns of focal amplification and rearrangements, resulting in concatemers present downstream from the integration sites. This suggests that concatemers of the host and viral genomes become amplified in tandem and are reinserted back into the host genome.[22] Moreover, this may explain extrachromosomal virus-host fusion episomes that can arise when looping integration occurs without reinsertion.[21]

Integration of the viral genome into the host genome often leads to deletion or truncation of the viral gene E2, resulting in loss of E2 transcript production. This in turn facilitates deregulated transcription of the viral E6 and E7 oncogenes, leading to ubiquitous expression of the corresponding E6 and E7 proteins.[21] Subsequently, this leads to deregulation of many cellular processes, including cell proliferation and apoptosis, for example by inactivation of the tumor-suppressors p53 and pRB.[1-4,7-10] Despite this knowledge, it is still unclear whether the integration of HPV into the human genome is associated with distinct biological consequences. Moreover, the association between HPV integration and poor patient outcomes is still debated, and results are controversial. Furthermore, tumors with a mixed viral physical status have been identified, posing the question whether or not these tumors show different biological behavior than tumors with solely integrated or episomal virus. This work aims to summarize the recent literature and adds to the knowledge of three reviews on HPV integration in HNSCC.[15,21,23]

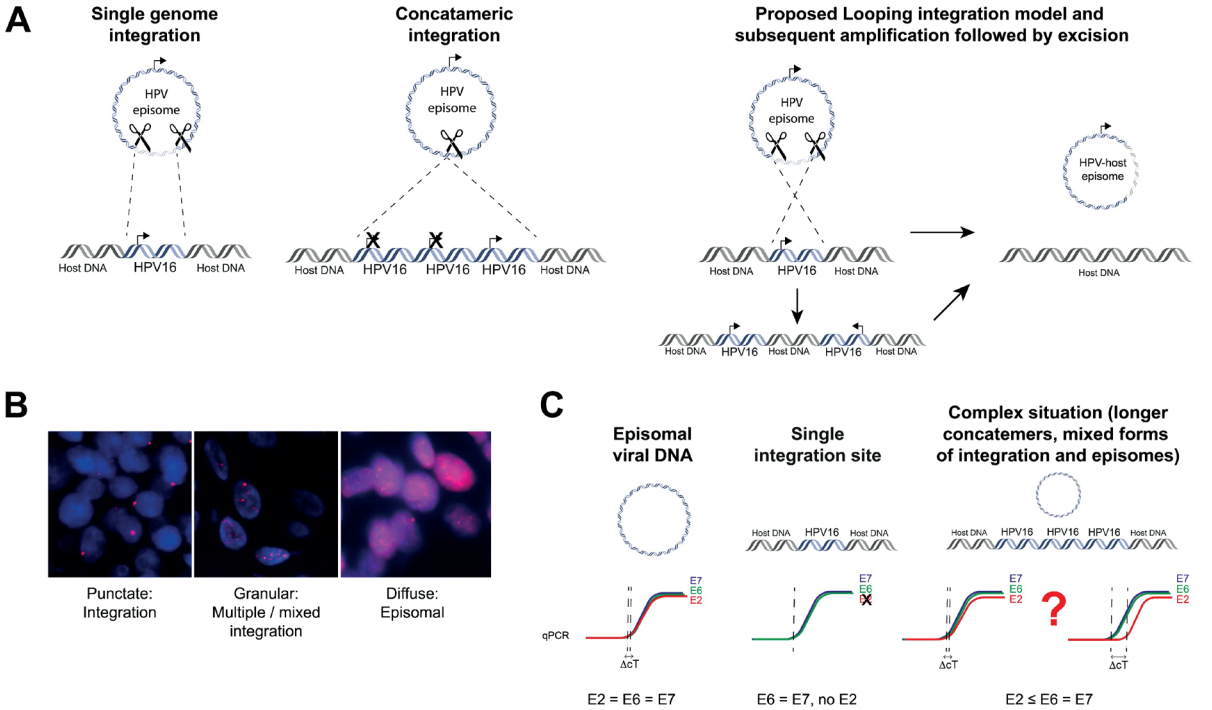

Figure 5.2 Mechanisms of episomal HPV-DNA integration into the human genome and non-sequencing based methods to prove integration. (A) Direct integration of a single viral genome into the host genome; direct integration of concatamerized viral genomes and proposed "Looping" integration of the viral genome with recurrent patterns of focal amplification and rearrangements next to the integration sites which finally may lead to excision and loss of viral DNA or viral-human fusion episomes; (B) fluorescence in situ hybridization with probes against HPV16 of tumor cells depicting integrated, mixed episomal and integrated and episomal status, magnification 100x; (C) qPCR strategy to analyze viral integration. An E2/E6 copy number ratio ≠ 1 may indicate disrupted E2 and viral integration. However, concatamerized HPV-genomes and/or additional HPV-episomes with several full-length E2 copies together with a single disrupted E2 gene will be challenging to detect.

Materials and methods

To find relevant literature on the causes and consequences of HPV integration in HNSCC, a detailed search was performed in the PubMed database (https://pubmed.ncbi.nlm.nih.gov, accessed 5 July 2021) using the search terms indicated in Appendix 5A. The timeframe of this analysis was fixed, by including papers published between January 2016 and April 2021. This systematic search resulted in a total of 101 papers, which were evaluated by reading the abstract followed by the full text (H.B. and I.D.). Thirty-six papers were eventually included in this study because they contained information about the physical status of HPV (episomal, integration) and HNSCC. One paper was included by screening references of the selected papers. To provide information, advantages and disadvantages of techniques to detect viral integration, 11 additional papers were included from PubMed database using search terms describing the different techniques.

Results

Involvement of APOBEC mediated anti-viral defense in HPV integration

Besides known mechanisms that can lead to DNA damage as represented in Figure 5.1, recent literature has provided evidence that Apolipoprotein B mRNA-editing catalytic (APOBEC) polypeptides are likely involved in HPV integration. APOBECs represent a family of 11 DNA cytosine deaminases that are a vital arm of the innate immune response. They potently inhibit retrovirus, transposon, and DNA virus replication. APOBECs catalyze the deamination of cytidine in both DNA and RNA. Inappropriate APOBEC expression has been identified as a genomic mutator that can eventually cause cancer.[24] Kondo *et al.* have reported that APOBECA3A (A3A) or A3B (A3B) expressions are involved in replication inhibition and increases the number of double strand breaks.[24] This in turn induces genomic instability and causes favorable circumstances for viral integration. Moreover, they found that A3A can catalyze the hypermutation of viral E2 and further state that A3A-induced deamination may increase the chance of viral integration Furthermore, supporting the results of Kondo *et al*., it was observed that the expression of A3B was found to be significantly higher in HPV-positive HNSCCs than in HPV-negative HNSCCs.[25] This additionally suggests that the high A3B expression in HPV-positive HNSCCs can cause beneficial genomic conditions allowing HPV integration. In conclusion, this association between APOBEC induced mutational signatures and HPV suggests that an impaired antiviral defense is a driving force in HPV-positive HNSCCs.[25]

Approaches to detect HPV integration in tumor tissue

To date, several techniques have been used to detect HPV integration in tumor tissue. Initially, approaches included in situ hybridization (ISH) or fluorescence in situ hybridization (FISH), which could visualize HPV DNA or RNA as well as viral integration at the single cell level in cells and tissues (Figure 5.2B). Alternatively, PCR-based techniques have been developed, including quantitative PCR (qPCR), which determines E6/E7 copy numbers in relation to E2, Detection of Integrated Papillomavirus Sequences (DIPS) PCR which detects virus-human DNA sequences, and Amplification of Papillomavirus Oncogene Transcripts (APOT) PCR, which detects virus–human RNA transcripts (Figures 5.2C and 5.3).

In addition, Next Generation Sequencing (NGS) techniques have been coming of age, including Whole Genome Sequencing (WGS), Whole Exome Sequencing (WES), and RNASeq, all identifying HPV-human nucleic acid sequences (Figure 5.3).

Emerging techniques are being developed, investigating viral integration in combination with HPV sequences capturing utilizing HPV-specific custom-made RNA probes. This enables DNA enrichment for viral sequences, increasing the chance to find HPV integration. This enrichment step is followed by amplification and NGS.[17-19] Examples of emerging techniques to detect HPV integration are nanopore sequencing on DNA/RNA isolated from fresh frozen tissues, combining HPV capturing with long read sequencing, as well as Targeted Locus Amplification (TLA) on DNA isolated from FFPE tissues, combining HPV capturing with circularization of DNA fragments and amplification (Figure 5.3). An overview of all the currently used techniques to identify HPV integration, as well as their advantages and disadvantages, are given in Table 5.1.

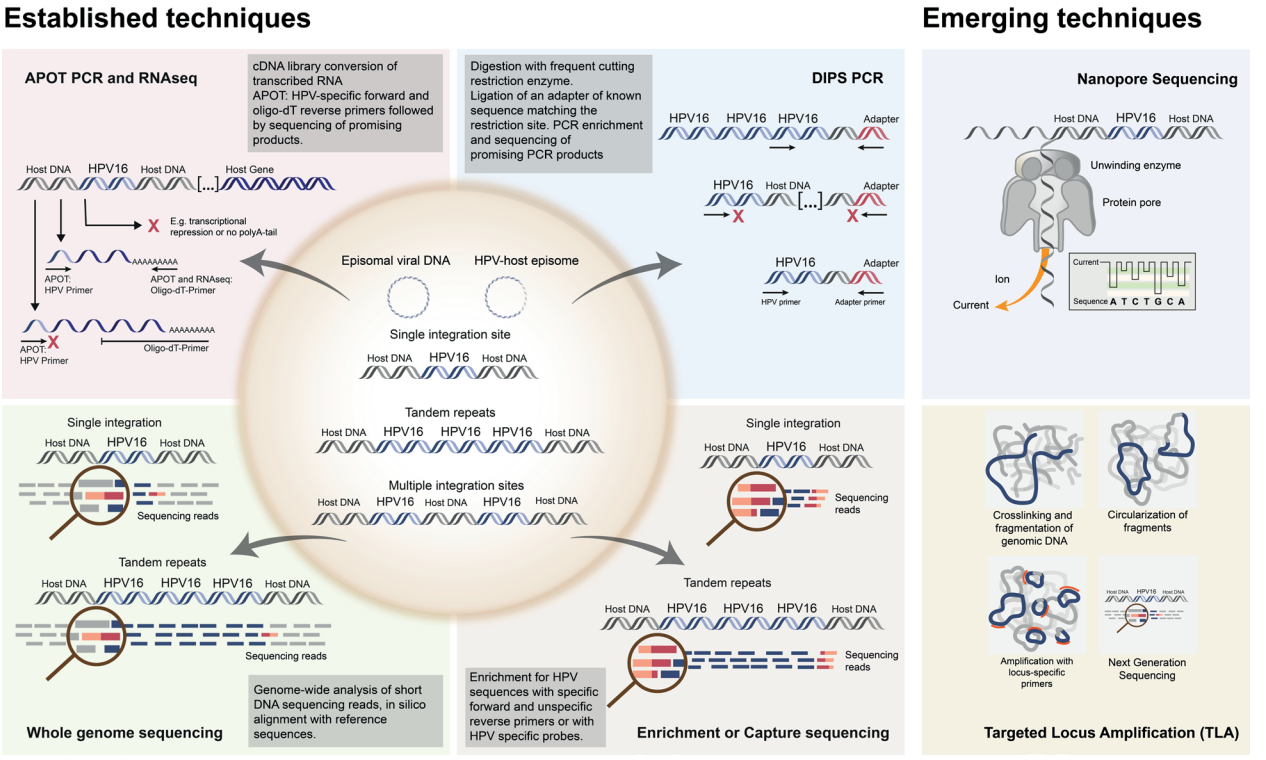

Figure 5.3 Overview of established and emerging techniques to detect HPV integration into the human genome. The established techniques to detect integration include RNA based techniques such as APOT PCR and RNAseq; DNA based techniques such as DIPS PCR, WGS, and Enrichment or Capture sequencing. Nanopore Sequencing and TLA are represented as emerging techniques for HPV integration detection. APOT = amplification of papilloma virus oncogene transcripts assay; DIPS-PCR = Detection of integrated papillomavirus sequences by ligation-mediated PCR; RNAseq = RNA sequencing.

Table 5.1 Advantages and disadvantages of techniques used to detect HPV integration.

Technique		Advantages	Disadvantages	Ref
In-situ hybridization (ISH)	(Fluorescence) in-situ hybridization ((F)ISH)	• Highly sensitive • Suitable for morphologically preserved isolated cells, histological tissue sections or chromosome preparations • Relatively fast results within one day • Relatively expensive with respect to PCR; relatively cheap with respect to sequencing • Able to identify number of integration sites per nucleus • Able to determine if integration site produces active transcripts (RNAse and DNAse pre-treatment)	• Requires prior knowledge about sequence of interest, e.g., in case of human–virus colocalization • Requires probe mixture to allow high-risk HPV detection, typing needs additional ISH experiment • Cannot determine site of integration if only virus probe is used • Cross-hybridization can occur when analyzing highly similar sequences (e.g., HPV6 and HPV11)	26,27
Polymerase Chain Reaction (PCR)	Quantitative or Real-Time PCR (qPCR, RT-PCR)	• Highly specific • Extremely sensitive • Suitable for fresh frozen material • Relatively cheap with respect to sequencing • Able to detect viral load based on fluorescence timing	• Less suitable for FFPE material • Cannot determine site of integration • Cannot indicate physical status • Cut-off for E2:E6/7-ratio is either less or strong discriminating • Integration can occur in different genes: E2 is not always deleted, E1 can also be deleted	28,29
	Detection of Integrated Papillomavirus Sequences PCR (DIPS-PCR)	• Suitable for fresh frozen material • Relatively cheap with respect to sequencing • Able to indicate physical status • Able to determine site of integration.	• Less suitable for FFPE material • Aimed only at fractures in E2 • Restriction enzyme is a limiting factor, since the site of integration into the human genome is unknown • Digested fragment needs to be at correct length: too long fragments make it difficult to be accurately detected by PCR, too short fragments ensures that integration site remains unknown	30–33
	Amplification of Papillomavirus Oncogene Transcripts PCR (APOT-PCR)	• Suitable for fresh frozen material • Relatively cheap with respect to sequencing • Able to indicate physical status • Able to determine integration site if integration occurred in a gene • Able to determine if integration site produces active transcripts • Highly accurate • Highly sensitive, even with large number of samples • Able to determine site of integration and viral copy number • Able to identify 5′ and 3′ end breakpoints through hybrid reads • Little to no bias due to nature of technique	• Less suitable for FFPE material • Requires stable RNA of good quality • Requires expression of active transcripts • Cannot determine site of integration if integration occurred in an intergenic region or an intron due to alternative splicing	30–33

Table 5.1 (continued)

Technique		Advantages	Disadvantages	Ref
Next-Generation Sequencing (NGS)	RNASeq	• Suitable for RNA from blood, fresh-frozen biopsy, FFPE, fine needle aspirates, core needle biopsies and single cells • Able to deep profile the transcriptome • Able to determine if integration site produces active transcripts • Requires lower depth to find 3′ HPV breakpoints with respect to DNA-based NGS due to level of virus transcripts • Unbiased approach to view entire RNA population	• Cannot find 5′ ends of HPV breakpoints • Cannot find HPV integrants that are transcriptionally repressed • Can produce false 3′ calls with splice reads • Depth may be reduced because of breadth of coverage	21,26
	Whole Genome Sequencing (WGS)	• Suitable for genomic DNA from blood and fresh-frozen biopsy. • Highly accurate • Highly sensitive, even with large number of samples • Able to determine site of integration and viral copy number • Able to identify 5′ and 3′ end breakpoints through hybrid reads • Little to no bias due to nature of technique	• Requires high read depth, deep sequencing and good coverage to find absolute integrant breakpoints • Relatively expensive with respect to PCR and (F)ISH • Relatively time consuming • Cannot determine if HPV integrants are transcriptionally active	21,34,35
	Whole Exome Sequencing (WES)	• Suitable for genomic DNA (gDNA) from blood, fresh-frozen biopsy • Highly accurate • Extremely sensitive, even with large number of samples • Relatively cheap with respect to WGS due to limited target • Able to obtain higher depth with respect to WGS due to limited target • Able to determine site of integration and viral copy number • Able to identify both 5′ and 3′ end breakpoints through hybrid reads • Little to no bias due to nature of technique	• Less suitable for FFPE material • Requires high read depth, deep sequencing and good coverage to find absolute integrant breakpoints. • Cannot identify integration sites in non-coding regions. • Cannot determine if HPV integrants are transcriptionally active	21,34,35
	Capture-based assay	• Suitable for genomic DNA and/or RNA from blood, fresh-frozen biopsy, DNA/RNA from FFPE, needle aspirates, and needle biopsies. • Able to determine site of integration and viral copy number • Able to identify 5′ and 3′ end breakpoints through hybrid reads • Increases chance of finding HPV integration sites due to capture • Little to no bias due to nature of technique • Can be adapted for additional methods, e.g. chromosome conformation	• Requires high read depth, deep sequencing and good coverage to find absolute integrant breakpoints • Requires individual probes for each HPV type • Cannot determine if HPV integrants are transcriptionally active • Excludes majority of host sequence	21,36

Table 5.1 (continued)

Technique		Advantages	Disadvantages	Ref
Emerging Techniques	Nanopore Sequencing	• Imaging equipment is not required; hence the system can be scaled down to portable level • On comparison to other massively parallel sequencers, the device is of much lower cost • The captured DNA can be sequenced rapidly • Long reads of DNA can be sequenced • Able to sequence long repetitive DNA sequences / structural variants	• Less suitable for FFPE material • Not suitable for single nucleotide variation detection • Extremely high molecular weight DNA needed for library preparation • The sequencer has the drawback of having high error rate ranging from 5% to 20%, based on the sort of molecules and methods of library preparation	37
	Targeted Locus Amplification	• Suitable for purified genomic DNA from fresh-frozen tissues, fresh tissues and FFPE material • Does not require detailed knowledge on locus sequence information • Able to determine site of integration and viral copy number • Able to identify both 5′ and 3′ end breakpoints through hybrid reads • Increases chance of finding HPV integration sites due to capture • Relatively long reads of DNA can be sequenced (1 kb in FFPE up to 50–100 kb in fresh cells) surrounding a known/specific sequence/captured target enabling more robust analysis with respect to traditional/standard DNA-based NGS.	• Requires high read depth, deep sequencing and good coverage to find absolute integrant breakpoints • Complex and extensive integration profile may be challenging to map out completely. • Integration sites could be missed in case of a large number of episomal HPV • Requires individual probes for each HPV type • Cannot determine if HPV integrants are transcriptionally active • Excludes majority of host sequence	38

[1] FFPE = formalin fixed paraffin embedded.

As mentioned above, an increasing number of studies have employed NGS techniques to determine the presence and location of the HPV integration in the human host genome. Inherent to reliable NGS data is an optimal bioinformatic pipeline that ensures rapid and exclusive detection of the viral genome from the large-scale genome-wide DNA sequencing of the cancer genome, typically by detecting virus-host chimeric fusions or paired-end reads.[21] Various bioinformatical approaches to identify viral integration sites have been described in the literature, including VirusSeq, VirusFinder, SurVirus, VirTect, HIVID2, and HGT-ID, which have been used to detect integrated HPV genomes specifically.[39-49] The variety of viral integration detection software tools might at least partly explain the broad range in the number of reported HPV integration sites (0–600) in cervical cancers.[22,50,51] It has been suggested that these high integration rates are a result of a low-stringency bioinformatics approach.[21] When mapping integration sites, multiple aspects that may induce artifacts in bioinformatic data should be considered. For example, splicing from within the HPV genome into the distal host genome could result in a fusion transcript, which can be misidentified as a breakpoint. In addition, sequencing machine contamination could lead to overestimation of HPV integration sites and bioinformatic tools may not be able to differentiate between reads from circularized (episomal) sequences and linearized genome sequences. Furthermore, artifacts could be introduced due to microhomology sites, duplicate reads, mitochondrial genomes integrating in a highly similar manner as human genomic DNA, and mismatch bases. Hence, there is a necessity for quality control of the bioinformatics data and confirmation of integration sites by other established techniques.[21,52] As a consequence, newly developed bioinformatic tools have recently been described in the literature, of which some examples will be explained below. Viral integration and Fusion identification (ViFi) has been presented as a new tool in detecting viral integrations from WGS data and human–virus fusion mRNA from RNAseq data. Unlike other bioinformatic pipelines that only use reference-based alignment mapping to identify viral reads, ViFi combines this with a phylogenetic model of HPV families to better detect evolutionarily divergent viruses.[53] An approach that detects Virus integration sites through Reference Sequence customization (VERSE) was first described in 2015 and is designed to 'correct' human reference genomes to create a new 'personalized' human reference genome, which aims to improve alignment of short reads and thereby virus detection sensitivity through WGS, RNAseq, and targeted sequencing.[25,54] A number of capture-based sequencing methods have been reported with bioinformatics tools. For example, nanopore sequencing distinguishes itself from other sequencing techniques as it enables sequencing of extremely long DNA molecules. This is at the cost of less sequence accuracy and the inability to sequence relatively short DNA and RNA isolated from FFPE material (Table 5.1). Specifically designed bioinformatic methods are being developed to

analyze the entire ultra-long sequencing reads and to perform error correction of the sequence data.[36] Furthermore, a novel pipeline, specifically for targeted capture sequencing data, has been generated, referred to as SearcHPV.[55] It has shown to operate in a more accurate and efficient manner than existing pipelines on capture sequencing data, something which has been lacking in the field. Another advantage of this software is that it performs local assembly of overlapping DNA segments around the junction site, which simplifies confirmation experiments. Cameron *et al*. developed a virus-centric approach, called VIRUSBreakend. This tool uses single breakends, breakpoints in which only one side can be unambiguously placed to the reference genome, with the advantage that viral integration can be detected in regions of low mappability, such as centromeres and telomeres. VIRUSBreakend first identifies the viral genome within the host genome, compares this to viral NCBI taxonomy IDs, selects a viral reference genome based on sequence similarity, and aligns all read pairs with this viral reference genome. Subsequently, single breakends are assembled and host integration sites are identified.[56]

Prevalence of HPV integration

Uterine cervical SCCs are HPV-positive in 95–100% of the cases with varying frequencies of integration for different HPV subtypes. HPV16 tends to integrate in 50–80% of the cases and HPV18 in >90%.[15,21,32] In OPSCCs, HPV positivity ranges from 20–90% in different studies depending on geographical location, sample preparation, and detection method used, and, furthermore, 90–95% of virus-positive OPSCCs are infected with HPV16.[44,57] Using FISH with whole virus genome probes, HPV integration percentages of 40–60% were described for OPSCCs.[58] An integration incidence of 40–100% was reported in tonsillar squamous cell carcinomas (TSCC)s using DIPS and APOT PCR techniques.[32,59] Recent literature describing E2, E6/E7 qPCR based HPV integration detection shows lower integration percentages (5–25%), dependent on anatomical tumor location, and a larger proportion of tumors containing both integrated and episomal HPV DNA (40–85%).[37,50-52,58,60,61] Integration rates determined with NGS-based techniques range from 15% to 70%.[60,62] However, the number of included patients is often low and the majority of studies included tumors originating from multiple locations, also outside the oropharynx. In addition, often no distinction is made between solely integrated HPV and the mixed form, in which episomal DNA is also present. These aspects, among others, make it difficult to directly compare studies and observed integration rates. Furthermore, differences in applied bioinformatic pipelines to detect viral integration might also contribute to divergent integration rates, as mentioned before.

Low HPV copy numbers are associated with integration in liquid biopsy

Recent research has shown that HPV DNA can also be efficiently detected in liquid biopsies (blood plasma, saliva), as part of the cell free DNA (cfDNA) fraction, and it is a promising biomarker for detection of early primary OPSCCs especially in groups of high risk patients.[63] cfDNA comprise DNA fragments of 160–180 base pairs, released in the blood by processes including apoptosis, necrosis, and secretion. Up to 0.1–1% of this cfDNA may consist of circulating tumor DNA. Plasma circulating tumor HPV-DNA (ctHPVDNA) can be measured over time to analyze the response of the tumor during cancer therapy using multianalyte digital PCR assays. Chera *et al*. investigated whether ctHPVDNA levels were associated with tumor HPV copy number and HPV physical state using digital droplet PCR.[64] In this study, the prevalence of HPV was observed in 44 patients from a total of 103 patients with OPSCC. HPV status was unknown in 49 patients though all tumors were $p16^{INK4A}$ positive. Their results show that low baseline levels of ctHPVDNA (≤200 copies/mL) were significantly associated with lower tumor HPV copy number (p=0.04). In addition, low tumor HPV copy number (≤5 copies/haploid genome) was significantly associated with HPV integration (p=0.02). From this, it can be concluded that low base-line levels of ctHPVDNA are indicative for low tumor HPV copy number and a greater probability of HPV integration. However, in this study, only 8 out of 20 HPV16-positive patients showed viral integration. Further studies are required to investigate this correlation in a larger sample size and/or the possibility to detect HPV-human DNA fusions in plasma derived cfDNA by NGS. Similarly, Tang *et al*. investigated whether HPV integration could be detected in saliva of OPSCC patients using qPCR analysis. They found a significant association between salivary HPV16 load (>10 copies/50 ng) and advanced disease stages.[59] Moreover, they identified mixed or fully integrated HPV in the saliva of 4 out of 127 OPSCC patients of which 74 patients harored HPV16 DNA and 89 patients showed $p16^{INK4A}$ staining. Even though this number is small and no correlation with disease stage was observed, the authors suggest that these results should be analyzed in a larger cohort.

Loci of HPV integration in the human genome

Molecular studies have provided evidence that ≥1 integration site (s) can be detected in HPV-positive cancers, including HNSCC.[15,65] HPV integration sites are distributed all over the human genome and often lie within or close to fragile sites. HPV integration hotspots have been found in chromosome 2q22.3, 3p14.2, 3q28, 8q24.22, 9q22, 13q22.1, 14q24.1, 17p11.1, and 17q23.1–17q23.2.[65,66] Interestingly, Walline *et al*. investigated if integration sites differed for oropharyngeal tumors comparing 10 HPV16 positive patients including five patients who responded well to therapy and five patients whose

tumor persisted and recurred.[67] They found that, in responsive tumors, HPV often integrates in intergenic regions, whereas recurrent tumors exhibited complex HPV integration patterns in cancer-associated genes. HPV integration is most frequently detected in genic regions, most often cancer-related genes, such as oncogenes (e.g., *TP63*, *MYC*, *ERBB2*) or tumor suppressor genes (e.g., *BCL2*, *FANCC*, *HDAC2*, *RAD51B*, *CSMD1*) and to a lesser extent in miRNA regions.[21,23] For example, Parfenov *et al.* studied 279 HNSCC samples in which 35 patients were high risk HPV positive. They observed HPV integration in a known gene among 54% of HPV-positive OPSCC, and 17% within 20 kb of a gene.[60] Similarly, Olthof *et al*. analyzed 75 HPV16 OPSCC samples and identified 37 integration sites in 29 OPSCC, of which 27 were in known or predicted genes, including 17 with a known role in tumorigenesis.[32] Based on these data, amongst others, it is suggested that HPV integration is not simply a random event, but rather prefers less protected and more accessible chromosomal regions, including highly transcribed (cancer) genes.[15]

An interesting finding using HPV integration detection for studying the clonal relationship between bilaterally developing TSCCs was reported by Pinatti *et al*.[68] In a case study, six integration events were detected by DIPS-PCR, including two intragenic events in the genes *CD36*, involved in fatty acid import and *LAMA3*, involved in cell adhesion, migration and differentiation of keratinocytes. No identical integration sites were observed between the left and right TSCC. However, it is remarkable that both TSCCs contained HPV16 integration in *CD36*, although slightly different with respect to the genomic location, i.e., intron 5 vs. intron 6. Although the authors suggested this finding as one of the events pointing to a clonal relation between both TSCCs, further mutational profiling of cellular genes and transcripts and access to samples other than FFPE tissue with better quality DNA/RNA are required to provide more evidence for the clonal nature of both TSCCs.

Consequences of viral integration

Deregulated viral gene expression

Based particularly on cell transfection studies, the general view is that, upon viral integration, the viral episome is most frequently opened in the E2 open reading frame. This often leads to deletion of E4 and E5 and part of E2 and L2.[13,14,66] Deletion of E2 disrupts its transcriptional repressor function in the viral Long Control Region (LCR), leading to upregulation of E6 and E7 and subsequent deregulation of cell signaling pathways, increased cellular proliferation and inhibition of apoptosis.[11,21] Interestingly, Reuschenbach *et al*. found from a total of 57 patients with HPV-positive OPSCC that 16

samples with undisrupted E2 are associated with methylation of E2 binding sites (E2BS3 and E2BSx4) in the LCR, leading to loss of protein expression, pointing to the same effect as deletion of the E2 gene. In most of the latter cases, the LCR was not methylated.[69]
More recent studies reported that viral genome methylation is not per se associated with HPV physical status. Although hypermethylation within the LCR was reported in two cell lines (UM-SCC-47 and CaSki), two other cell lines (UM-SCC-104 and SiHa) with a mixed physical status of the HPV genome contained a unmethylated LCR.[70] In this respect, Hatano *et al*. observed that the methylation status of the integrated HPV genome in three HNSCC cell lines (UPCI:SCC090, UPCI:SCC152, and UPCI:SCC154) correlated to the methylation status of the host genome flanking the integration breakpoints.[71] As a consequence, they suggested that viral (onco)gene expression might be dependent on the location of integration. Nevertheless, multiple studies on primary tumors have shown that disruption of E2 upon viral integration will not per se lead to increased expression of E6 and E7 oncogenes, suggesting that constitutive rather than high-level expression of viral oncogene transcripts is required in HPV induced carcinogenesis. In tumors with episomal HPV, constitutive expression of E6 and E7 has also been reported.[2,58,72–75]

Deregulated human gene expression

Besides the effects on viral oncogene expression, HPV integration might also directly or indirectly affect the host genome. Direct involvement of viral integration on human gene expression may occur when the virus is integrating in or adjacent to a cancer gene, thereby (in)activating its expression. Integration in a tumor suppressor gene might result in loss of gene function, with loss of the wildtype gene on the other chromosome, or translation of truncated proteins. Integration adjacent to an oncogene could lead to gene amplification or enhanced expression from the viral promotor. Additionally, intra - or interchromosomal rearrangements followed by altered expression of genes in these regions might occur. Figure 5.4A-C shows a number of examples of reported genes directly affected by viral integrants.[8,15,22,23,60] Alternatively, human gene expression may be indirectly deregulated by ubiquitous E6 and E7 expression, independent of HPV physical status. Figure 5.4D shows reported examples and consequences of indirect deregulation of cellular pathways and processes by HPV infection. Below, examples from the recent literature are described.

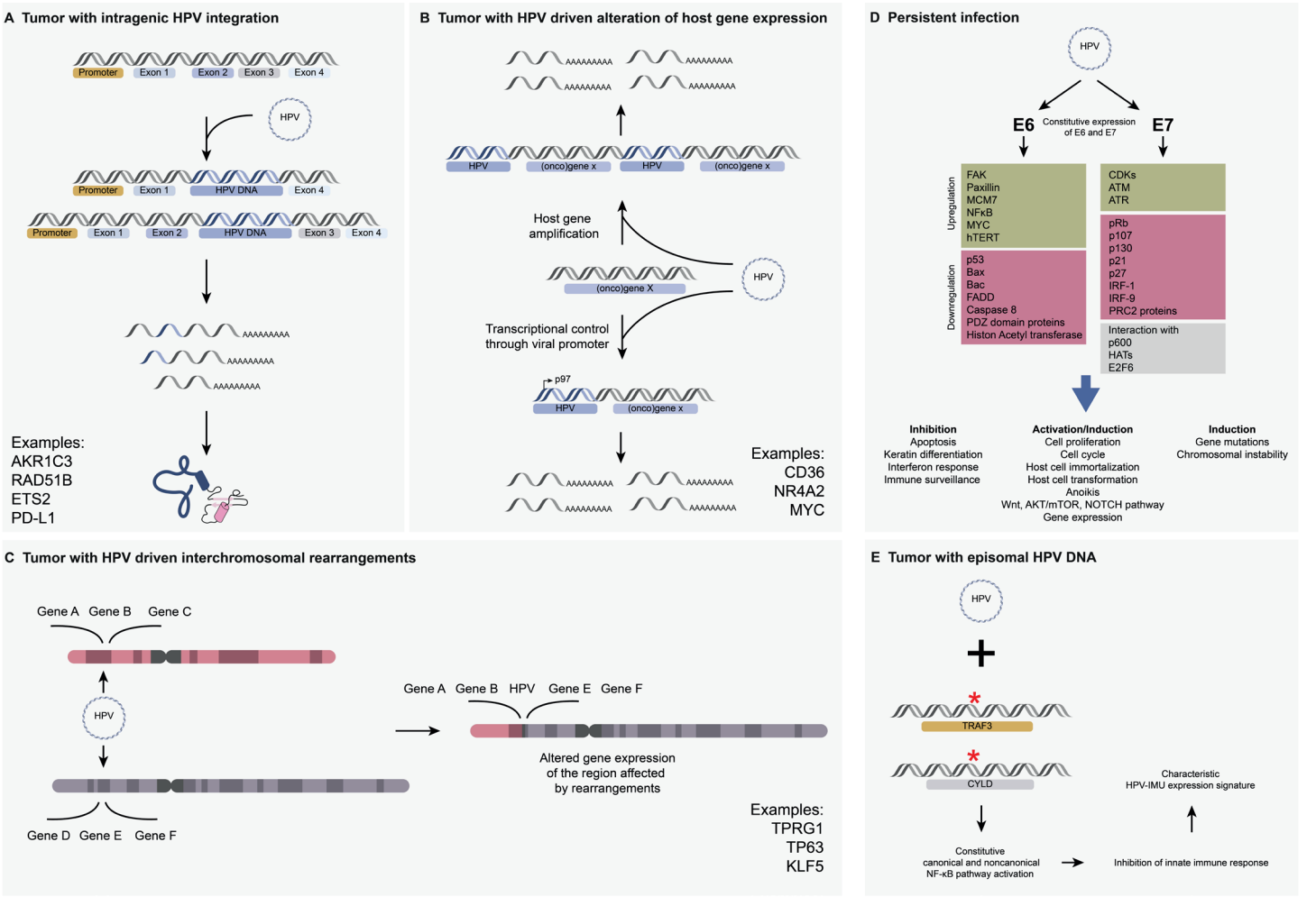
A Tumor with intragenic HPV integration
Promoter
Exon 1
Exon 2
Exon 3
Exon 4
HPV
HPV DNA
AAAAAAAAA
Examples:
AKR1C3
RAD51B
ETS2
PD-L1
B Tumor with HPV driven alteration of host gene expression
HPV
(onco)gene x
Host gene amplification
(onco)gene X
Transcriptional control through viral promoter
p97
Examples:
CD36
NR4A2
MYC
C Tumor with HPV driven interchromosomal rearrangements
Gene A
Gene B
Gene C
Gene D
Gene E
Gene F
Altered gene expression of the region affected by rearrangements
Examples:
TPRG1
TP63
KLF5
D Persistent infection
E6
E7
Constitutive expression of E6 and E7
Upregulation
Downregulation
FAK
Paxillin
MCM7
NFκB
MYC
hTERT
CDKs
ATM
ATR
p53
Bax
Bac
FADD
Caspase 8
PDZ domain proteins
Histon Acetyl transferase
pRb
p107
p130
p21
p27
IRF-1
IRF-9
PRC2 proteins
Interaction with
p600
HATs
E2F6
Inhibition
Apoptosis
Keratin differentiation
Interferon response
Immune surveillance
Activation/Induction
Cell proliferation
Cell cycle
Host cell immortalization
Host cell transformation
Anoikis
Wnt, AKT/mTOR, NOTCH pathway
Gene expression
Induction
Gene mutations
Chromosomal instability
E Tumor with episomal HPV DNA
TRAF3
CYLD
Characteristic
HPV-IMU expression signature
Constitutive
canonical and noncanonical
NF-κB pathway activation
Inhibition of innate immune response

Figure 5.4 Direct and indirect consequences of HPV infection on human gene expression. (A) Integration of HPV in intragenic regions of the human genome causing loss of gene function and/or truncated proteins e.g., *AKR1C3*, *RAD51B*, *ETS2* and *PD-L1*[22,32]; (B) integration of HPV near proto-oncogenes such as *CD36*, *NR4A2* and *MYC*, leading to oncogene activation, such as gene amplification or upregulation of gene expression[23]; (C) HPV integration may lead to interchromosomal rearrangements, amplification of genes and subsequent increase in expression of genes such as *TP63*, *TPRG1* and *KLF5*[22,32,60,83]; (D) The constitutive expression of E6 and E7 oncoproteins upon HPV infection (independent of physical status) will lead to deregulation of cell signaling pathways, inhibition of apoptosis, activation of cell proliferation and induction of gene mutations or chromosomal instability[11,96]; (E) Tumors harboring episomal HPV often show the presence of *TRAF3*/*CYLD* mutations leading to constitutive activation of NF-иB, resulting in inhibition of innate immune responses, which is a characteristic of HPV-immune response and mesenchymal cell differentiation (HPV-IMU) signature types.[92]

Deregulated expression of the targeted gene by HPV integration

Hassounah *et al*. showed that HPV is able to integrate into the *CD274* gene encoding Programmed Death Ligand 1 (PD-L1), specifically in front of the sequence coding for the transmembrane domain of the protein (within the intron after exon 4).[76] This results in transcription of a truncated isoform of PD-L1 that is unable to bind to the membrane but is rather secreted by the cell, as confirmed in vitro using cell lines and transfection experiments. The truncated isoform of PD-L1 maintains its ability to bind to PD-1, inducing a negative regulation of T cell function outside of the cell, which was confirmed by inhibition of IL-2 and IFN-γ secretion. Additionally, Koneva *et al*. also identified three tumors in which *CD274* was used as an HPV integration site (integrations within intron 4 and two 'enhancer sites' upstream of *CD274)*, which correlated with upregulated PD-L1 expression.[77] Broutian *et al*. observed HPV insertions flanking a 16-fold somatic amplification of the gene *PIM1* (Proviral insertion site for Moloney murine leukemia virus MuLV) in the HNSCC cell line UPCI:SCC090, in which more integration sites have been identified.[8,22] This amplification was accompanied by an increase of *PIM1* transcripts.[78] PIM1 overexpression has been identified in HNSCCs and has been associated with poor survival.[79-81] PIM kinases are involved in cellular transformation and substrates of PIM kinase phosphorylation are involved in cell cycle progression, cell growth, and cell death. PIM1 activation causes phosphorylation of several substrates of the PIK3CA/AKT/mTOR pathway, which in turn promotes an increased activation of this pathway and allows increased cell metabolism and growth.[78] A case report published by Huebbers *et al*. describes a very rare malignant transformation of juvenile-onset recurrent respiratory papillomatosis of the larynx.[82] They reported that the tumor contained integration of low-risk HPV type 6 in the Aldo-Keto Reductase 1C3 (*AKR1C3*) gene, deletion of the corresponding chromosomal region 10p14–10p15.2, and loss of AKR1C3 protein expression.[83]

Deregulated expression of human genes by HPV integration

Huebbers *et al*. investigated differences in human gene expression between oropharyngeal tumors with and without HPV integration (detected by APOT/DIPS PCR).[30] They showed that AKR1C1 and AKR1C3 protein expression was upregulated in OPSCC with HPV integration. Upregulation of AKRs (compared to expression in the adjacent normal squamous epithelium) was also detected in HPV-negative OPSCC, most probably because of oxidative stress response, induced by mutations in the Keap1/Cul3/NRF2 system.[30,84] AKRs play a role in prostaglandin, steroid hormone, and retinoid metabolism. Furthermore, they are phase I detoxifying enzymes involved in the modification of chemotherapeutic drugs.[83] Interestingly, there are feedback loops between oxidative stress response and AKR1C expression with NRF2 binding to antioxidant response elements (ARE) in the promoter regions of the AKRCs increasing their expression.[83] Furthermore, the viral spliced isoform HPV16-E6*I was shown to interact with SP1-binding sites within the AKR1C1 promoter regions also resulting in increased AKR1C1 expression.[84] On the other hand, an increase in AKR1C1 and AKR1C3 protein expression results in decreased concentrations of retinoic acids, known inhibitors of NRF2 function, which subsequently also lead to NRF2 activation.[85] The activation of NRF2 consequently activates PI3K-AKT signaling, metabolic reprogramming, cell proliferation, insufficiency in autophagy, chemotherapy resistance as well as impaired DNA damage response.[30,86,87] It was also demonstrated by Huebbers *et al*. and Zhang *et al*. that HPV16-E6*I expression was upregulated significantly in OPSCCs with integrated viral genome.[30,86] Furthermore, in both of these studies, viral integration and E6*I overexpression are correlated with keratinocyte differentiation signatures. Similarly, Paget-Bailly *et al.* reported that ectopic expression of HPV16 E6*I induced deregulation of cellular genes participating in ROS metabolism, promoting viral integration by inducing genome instability.[88] The presence of E6 partially counteracts the impact of E6*I. Additionally, the above is also supported by studying a clinical cohort, where the subgroup of tumors overexpressing E6*I was associated with key cancer pathways linked to ROS metabolism.[89] However, further studies should be performed to understand how E6*I regulates genes associated with oxidative stress and how this impacts HPV-driven tumorigenesis.[88]

Pannone *et al*. showed an association between HPV integration (detected by ISH) and Toll like receptor (TLR) 4 downregulation.[90] TLRs are predominantly involved in the innate immune response to pathogens including HPV and recognize Pathogen-associated Molecular Patterns (PAMPs) such as nucleic acids or proteins of viral origin, which serve as TLR activating ligands.[91] Ligand bound TLR4 then triggers lipid raft flowing, resulting in a conformational change. This in turn leads to aggregation of NADPH oxidase subunits on these lipid rafts resulting in ROS production and increased HIF1α

expression adding to the hypoxic tumor conditions.[91] TLR4 furthermore activates signaling cascades including tumor necrosis factor receptor-associated factor 3 (TRAF3) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-ϰB), which regulate the production of interferons (INF), inflammatory cytokines, and chemokines. However, in uterine cervical carcinomas and HPV-positive OPSCCs, a decrease in the TLR4 expression compared to normal epithelium is observed.[90] The viral proteins E6 and E7 have the property to interfere with innate immunity, e.g., by interacting with interferon regulator factor 3 (IRF-3) (E6) or IRF-1 (E7). As a result, HPV gains the ability to escape both innate and adaptive immune response and further avoid being recognized by Antigen Presenting Cells (APC)s.[90]

The presence of episomal HPV DNA also showed to correlate with deregulation of pathways involved in immune response and cell survival in an indirect manner. Hajek *et al.* discovered that 85% of tumors with mutations in the genes *TRAF3* and *CYLD* (Cylindromatosis Lysine 63 Deubiquitinase) contained episomal HPV (data from The Cancer Genome Atlas).[92] *TRAF3* is one of the most frequently mutated genes in HPV-positive HNSCCs (25% of HPV-positive tumors), but, remarkably, is not usually found to be mutated in their HPV-negative counterparts (2%).[93] In addition, the tumor suppressor gene *CYLD* was found to be mutated in 11% of HPV-positive tumors. Both TRAF3 and CYLD play a role in both negatively regulating NF-ϰB canonical and noncanonical pathways while simultaneously stimulating a potent and first-line antiviral response through type I IFN signaling. Mutations in these genes will therefore lead to constitutive activation of NF-ϰB, which promotes cell survival and an impaired innate immunity against viral infections.[68,94,95] Moreover, it is suggested that maintenance of episomal HPV even pressures cells to mutate *TRAF3*/*CYLD*. These mutations might provide support for an alternative mechanism of HPV tumorigenesis in HNSCCs, not depending on viral integration into the host cell genome, to provoke a malignant transformation.[92]

Subgroups of HPV-positive tumors associated with viral integration status

Recent studies have shown that HPV-positive tumors represent a heterogeneous group with respect to mRNA expression signatures as well as HPV integration status, with biological and clinical relevance. Two main subgroups have been characterized based on mRNA expression signatures, namely HPV-IMU and HPV-KRT (HPV-keratinocyte differentiation and oxidative reduction process).[2,86,97] Molecular analyses revealed that the HPV-KRT subgroup more frequently contains integrated HPV (70–78% of the cases), shows a lower expression of E2/E4/E5, and has a higher ratio of spliced E6 compared to full length E6, which is in agreement with observations described above. Furthermore, this group was enriched for chromosome 3q amplifications and PIK3CA mutations. HPV-

IMU tumors showed less integration (25–36% of the cases) and were enriched for chromosome 16q losses (detected by RNA sequencing).

Another study of Locati *et al*. identified three main clusters of HPV-positive tumors; Cl1 (immune-related), Cl2 (epithelial-mesenchymal transition-related), and Cl3 (proliferation-related).[98] Tumors classified as Cl1 showed viral integration in 45% of the cases, whereas tumors classified as Cl2 and Cl3 showed 100% and 77% integration, respectively. In addition, the three clusters have been observed to have prognostic relevance, with Cl1 correlating to the best survival rate, and Cl2 to the worst survival rate. Knowledge on subtypes within HPV-positive tumors might contribute to patient selection for either de-escalation or personalized therapeutic approaches.[11]

HPV integration in relation to prognosis

The association of HPV integration with patient prognosis has been a topic of debate for several years.[15] More recent studies indicate an association of viral integration with unfavorable prognosis.

Nulton *et al.* demonstrated, using the expression of E2 as a marker for integration in TCGA HNSCC samples, that patients with fully episomal or a mixed form of HPV16 showed better survival than patients with integrated HPV16 as well as patients with HPV-negative HNSCCs.[99] Similarly, Hajek *et al*. observed that the HPV-positive subset of HNSCC in the TCGA database with mutations in the genes TRAF3 and CYLD were associated with the maintenance of episomal HPV and improved survival of patients.[92] For this association, they used the NGS determined integration data from the study of Parfenov *et al*.[60] Moreover, Veitía *et al*. evaluated 80 fresh biopsies of head and neck cancer, mostly oral cavity, larynx, and oropharynx tumors, using E2/E6 qPCR. Of the 28 HPV16 positive samples, 86% displayed integration, possessed low viral load and correlated to poor prognosis.[100] Supporting these results, Koneva *et al*. showed that patients with (RNASeq determined) integration-positive oropharyngeal and oral cavity tumors had statistically significant worse survival than patients with integration-negative tumors and similar survival as patients with HPV-negative HNSCCs.[77] Moreover, patients with integrated HPV were significantly older than patients with episomal HPV and comparable to HPV-negative patients, suggesting that older age was associated with worse survival.[77,99]

In addition, Huebbers *et al*. showed that HPV integration in oropharyngeal tumors (analyzed with APOT- and DIPS PCR) was associated with upregulation of AKR1C1 and AKR1C3 expression.[30] Upregulation of AKR1C1 and AKR1C3 correlated with negative

outcomes for both chemo- and radiotherapy in both overall and disease-free survival. Contrastingly, low expression of AKR1C1 and/or AKR1C3 was significantly correlated with favorable outcomes in surgical treatment. Intriguingly, viral integration also seems to be associated with a more progressive and persistent disease.[101-103]

In contrast, both Vojtechova *et al*. and Lim *et al*. showed that there were no significant differences in survival between patients with episomal, mixed or integrated HPV16 in oropharyngeal tumors (*n*=186 and *n*=179, respectively).[104,105] Vojtechova used three different detection techniques (E2 transcript breakpoint analysis, APOT, and Southern blotting). Lim *et al*. observed a trend towards better survival in patients with mixed HPV compared to patients with either episomal or integrated HPV; however, they used E2/E6 qPCR, possibly leading to overestimation of mixed viral physical status, as discussed before.

Recently, Pinatti *et al*. showed, using DIPS-PCR analysis on 35 tumors, mainly of the oropharynx, that HPV integration was correlated with favorable disease-specific survival when compared to patients without integration.[106]

Overall, studies reporting on the correlation of viral integration with patient prognosis of HPV-positive HNSCCs have shown inconsistent results. As mentioned before, the technique used to detect viral integration is important to consider when interpreting the results of these studies. As an example, PCR for E2 and E6/7 expression might overestimate mixed physical status of HPV. Furthermore, studies often include tumors from different anatomical locations and relatively small patient groups.

Conclusions

In conclusion, a number of different technologies (including FISH, PCR, and NGS) have been used to determine the physical status of HPV in HNSCC, predominantly HPV16 in oropharyngeal tumors. Dependent on the viral detection strategy, HPV integration prevalence may differ. Results indicate that HPV integration is not simply a random event but rather prefers less protected and more accessible chromosomal regions, including highly transcribed (cancer) genes. Besides known mechanisms that can lead to DNA damage and subsequent viral integration, for example ROS, toxic agents, and inflammation, recent literature has provided evidence that APOBEC expression, induced by antiviral response, is doing so. Recent studies show that HPV integration affects both the viral and host genome, leading to constitutive expression of viral oncoproteins and

deregulation of cellular (cancer) genes, possibly conferring additional neoplastic pressure. HPV integration appears to upregulate genes involved in metabolic pathways and immune evasion and downregulate genes involved in inflammation, apoptosis, and immune responses. On the other hand, episomal HPV was associated with mutations in *TRAF3* and *CYLD*. Although new data suggest a correlation between HPV integration and unfavorable prognosis, more genome-wide studies with a larger sample size, especially of oropharyngeal origin, are required. Ideally, a uniform detection method utilizing NGS technology should be applied, and integration results should be validated using multiple techniques, to further investigate the biological and clinical implications of HPV integration in HNSCC.

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Appendix 5.A Search Terms Used for Systematic PubMed Search

((Head[Tiab] OR neck[Tiab] OR "head and neck" [Tiab] OR "head-neck" OR "head-and-neck" [Tiab] OR oral[Tiab] OR pharyn*[Tiab] OR OR laryn*[Tiab] OR oropharyn*[Tiab] OR nasopharyn*[Tiab] OR hypopharyn*[Tiab] OR throat[Tiab] OR glotti*[Tiab] OR mouth[Tiab] OR palate[Tiab] OR gingiva*[Tiab] OR lip[Tiab] OR cheek[Tiab] OR bucc*[Tiab] OR gum*[Tiab] OR tonsil*[Tiab] OR tongue[Tiab] OR nasal[Tiab] OR paranasal[Tiab] OR sinus[Tiab] OR saliv*[Tiab] OR ent[Tiab] OR aerodigestive[Tiab] OR "aero digestive" [Tiab] OR aero-digestive[Tiab])
AND (cancer* OR carcinoma* OR neoplas* OR tumor* OR tumour* OR malignan* OR SCC OR "Neoplasms"[Mesh])) OR (hnscc[Tiab] OR scchn[Tiab] OR "Head and Neck Neoplasms"[Mesh])
AND
("Human papilloma virus" [Tiab] OR "Human papilloma viruses" [Tiab] OR "Papillomavirus, Human" [Tiab] OR "Human papillomavirus" [Tiab] OR HPV [Tiab] OR HR-HPV [Tiab] OR "High-risk HPV" [Tiab] OR "HPV infection*" [Tiab] OR "Papillomavirus Infections/pathology" [Mesh])
AND
(integration [Tiab] OR "virus integration*" [Tiab] OR "virus integration" [Mesh] OR "Viral integration*" [Tiab] OR "human papillomavirus integration" [Tiab] OR "HPV integration" [Tiab] OR "genome integration" [Tiab] OR "viral DNA integration" [Tiab] OR "virus DNA integration" [Tiab] OR "HPV DNA integration" [Tiab] OR "HPV insertion*" [Tiab] OR "Human papillomavirus insertion*" [Tiab])

Chapter 6

Proximity ligation-based sequencing for the identification of Human Papillomavirus genomic integration sites in formalin-fixed paraffin embedded oropharyngeal squamous cell carcinomas

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Submitted

PART II

New therapeutic options and tumor-derived culture models

Chapter 7

The antiviral agent cidofovir induces DNA damage and mitotic catastrophe in HPV-positive and -negative head and neck squamous cell carcinomas in-vitro

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Cancers (Basel). 2019;11(7):919

Abstract

Cidofovir (CDV) is an antiviral agent with antiproliferative properties. The aim of our study was to investigate the efficacy of CDV in HPV-positive and -negative head and neck squamous cell carcinoma (HNSCC) cell lines and whether it is caused by a difference in response to DNA damage. Upon CDV treatment of HNSCC and normal oral keratinocyte cell lines, we carried out MTT analysis (cell viability), flow cytometry (cell cycle analysis), (immuno)fluorescence and western blotting (DNA double strand breaks, DNA damage response, apoptosis, and mitotic catastrophe). The growth of the cell lines was inhibited by CDV treatment and resulted in γ-H2AX accumulation and upregulation of DNA repair proteins. CDV did not activate apoptosis but induced S- and G2/M phase arrest. Phospho-Aurora Kinase immunostaining showed a decrease in the number of mitoses but an increase in aberrant mitoses suggesting mitotic catastrophe. In conclusion, CDV inhibits cell growth in HPV-positive and -negative HNSCC cell lines and was more profound in the HPV-positive cell lines. CDV treated cells show accumulation of DNA DSBs and DNA damage response activation, but apoptosis does not seem to occur. Rather our data indicate the occurrence of mitotic catastrophe.

Introduction

Each year ~600,000 people worldwide are diagnosed with head and neck squamous cell carcinoma (HNSCC), making HNSCC the sixth most common cancer in the world.[1] Important risk factors for the development of HNSCC are alcohol consumption and/or smoking as well as high-risk human papillomavirus (HPV) infections. HPV-positive HNSCC is considered to be a distinct clinical and molecular entity in comparison to HPV-negative HNSCC.[2] The mortality rates have hardly decreased over the last decades and the five-year survival rate still ranges between 40–50%, even though improvements in detection and treatment have been achieved.[3] The HPV status of the tumor possesses powerful prognostic value, where HPV-positive patients have a more favorable prognosis.[4,5] There is an urgent need for new agents that can be integrated into or replace current treatment regimens to improve outcome and quality of life of HNSCC patients.

Cidofovir (CDV) is an acyclic nucleoside phosphonate which targets DNA viruses that encode for their own DNA polymerase, because the active diphosphate metabolite (CDVpp) has a higher affinity for viral DNA polymerase compared to cellular DNA polymerase. CDVpp competitively inhibits the incorporation of deoxycytidine triphosphate (dCTP) into viral DNA by viral DNA polymerase, which results in reduction in the rate of viral DNA synthesis.[6,7] Currently, CDV is approved by the Food and Drug Administration for intravenous administration in the therapy of cytomegalovirus retinitis in AIDS patients.[8,9] CDV is also used off-label for the treatment of infections caused by other DNA viruses, including papilloma- and polyomaviruses. In earlier studies, CDV has shown to have anti-proliferative properties against HPV-positive cervical carcinoma and HPV-negative transformed cell lines.[10] CDV has also been reported to be effective in a number of HPV-negative malignancies in vivo, such as glioblastoma and nasopharyngeal carcinoma.[11,12] The effects of CDV on HPV-positive induced benign and malignant proliferations should be linked to the antiproliferative effects of the compound as HPV uses the host DNA polymerase for replication.[10,13] Today, the molecular mechanisms underlying the effectivity of CDV are not completely understood. One hypothesis is that the selectivity of CDV for HPV-transformed cells is based on differences in replication rate, CDV incorporation into the cellular DNA, and in response to DNA damage caused by CDV.[14] The aim of our study was to investigate the in vitro efficacy of CDV in HPV-positive and -negative HNSCC cell lines and the normal oral keratinocyte (NOK) cell line, which is immortalized by the activation of hTERT[15], and whether this efficacy is caused by a difference in response to DNA damage.

Methods

Cell lines and culture conditions

Three HPV16-positive head and neck squamous cell carcinoma (HNSCC) cell lines: UD-SCC-2 (from Thomas Hoffmann, University of Ulm, Germany), 93-VU-147T (Johan. P. De Winter, VU Medical Center, the Netherlands), and UM-SCC-47 (Thomas E. Carey, University of Michigan, Ann Arbor, MI, USA) were used. Two HPV16-negative HNSCC cell lines: UPCI-SCC-72 and UPCI-SCC-003 (both from Susanne M. Collins, University of Pittsburgh, Pittsburgh, PA, USA) were used. Two HPV16-positive uterine cervical carcinoma cell lines, SiHa and CaSki, were purchased from the American Type Culture Collection (ATCC). The normal oral keratinocyte (NOK) cell line (Karl Munger, Tufts University Medical School, Boston, MA, USA), which is immortalized by activation of h-TERT[15] is a cell line prepared from gingival tissues obtained from oral surgeries[16] as described previously.[17] Cells were cultured at 37 °C in a humidified atmosphere with 5% CO2. All HNSCC cell lines used in this study were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). CaSki was cultured in Roswell Park Memorial Institute (RPMI) with 10% FCS. SiHa was cultured in Minimum Essential Medium (MEM) with 10% FCS, supplemented with L-glutamine and non-essential amino acids. The NOK cell line was cultured in keratinocyte serum-free medium (KSFM) supplemented with (2.6 µg/mL) bovine pituitary extract (BPE) and (0.16 ng/mL) recombinant epidermal growth factor (rEGF). All the cell lines were regularly tested and found to be mycoplasma-free. All cell lines were confirmed to have unique genotypes, as tested using the ProfilerPlus assay.[18] The presence of HPV DNA was detected by PCR using the consensus primer set GP5+/6+.[19]

In vitro cell proliferation assay

Cells were seeded in 96-well flat bottom plates at densities that allowed exponential growth for the duration of the experiment. They were placed in the cell culture incubator overnight at 37°C allowing the cells to attach, after which they were treated with concentrations of Cidofovir (Vistide, Gilead Sciences Inc, Foster City, CA, USA) of 10, 100, 200, and 300 µM or PBS (control). At indicated time points post-treatment (3, 6, and 9 days), the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, Saint Louis, MO, USA) was performed as previously described.[20] The experiments were performed in triplicate.

Irradiation

The cells were irradiated at room temperature with 4 Gray (Gy). After 4 and 24 h of incubation the irradiated cells and the no irradiated control cells were fixed with methanol for 15 min at −20°C and analyzed for γ-H2AX expression by immuno-fluorescence (see below).

Cell cycle analysis

Cells were seeded in T25 culture flasks and placed in the cell culture incubator at 37°C and allowed to attach overnight. Culture medium was added containing CDV (IC50) or PBS. After 3 and 6 days, cells were washed with PBS and trypsinized to form a cell pellet. Ice-cold 70% ethanol was added to the cell pellet while vortexing, assuring fixation of the cells and minimizing cell clumping. Cells in 70% ethanol were stored at −20°C for a minimal duration of 30 min. Cells were washed with PBS and resuspended in 0.5 mL propidium iodide(PI)/RNAse staining solution (100 µg/mL PI and 1 mg/mL RNAse in PBS). Cells were incubated on ice for 30 min and analyzed by flow cytometry using a FACScanto II (BD Biosciences, San Jose, CA, USA). Data analysis was performed using FACSdiva software (BD Biosciences). The different cell cycle regions were set to those defined by the untreated control cells for each cell line individually.

Apoptosis assay

As a positive control for apoptosis, the cells were treated with 1 µM Staurosporine (Sigma-Aldrich). For the Annexin-V assay cells were seeded in 96-wells plates and allowed to attach overnight at 37°C. Cells were treated with CDV (IC50) or PBS for 3 and 6 days. Cells were stained with Hoechst 33,342 (200 µg/mL, Sigma-Aldrich) in culture medium for 15 min at 37°C. Cells were washed with Annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM CaCl2 in PBS) and stained with Annexin-V-FITC (2.5 µg/mL in Annexin-V binding buffer) for 15 min at 37°C. Staining intensities of cells were measured in High-Content Imaging. Data was acquired using a BDpathway855 High-Content Bioimager (BD Biosciences). Digitalization and segmentation of acquired data was done with Attovision software (BD Biosciences). Processed data was evaluated by DIVAsoftware (BD Biosciences).

Immunofluorescence staining of γ-H2AX, cyclin B1, and phospho-aurora kinase A/B/C

Cells were grown in 96-well plates (γ-H2AX) or on coverslips (cyclin B1 and phospho-Aurora Kinase A/B/C) and allowed to attach overnight at 37°C. Culture medium

containing CDV (IC50) or PBS was added, and cells were incubated at 37°C. After 3 and 6 days, cells were washed with PBS followed by fixation in CytoRich Red for 20 min at RT (γ-H2AX) or methanol for 15 min at −20°C (cyclin B1 and phospho-Aurora Kinase A/B/C). After washing with PBS, the cells were permeabilized with 0.1% Triton in TBS/T (0.1% Tween20 in TBS) for 20 min and then blocked with 5% bovine serum albumin (BSA) in TBS/T for 30 min at RT. Cells were incubated with the primary antibody (Table S7.1) diluted in blocking buffer overnight at 4°C. After washing with TBS/T, the cells were incubated with a fluorescent-labeled secondary antibody directed against the primary antibody (Table S7.1). For the quantification of γ-H2AX expression after CDV treatment, cells were stained with (200 µg/mL) Hoechst 33,342 for 10 min at 37°C. Staining intensities of cells were measured in High-Content Imaging. Data was acquired using a BDpathway855 High-Content Bioimager (BD Biosciences). Digitalization and segmentation of acquired data was done with Attovision software (BD Biosciences). Processed data was evaluated by DIVAsoftware (BD Biosciences).

For cyclin B1, phospho-Aurora kinase A/B/C, and for γ-H2AX expression in the radiotherapy experiment, nuclear morphology was visualized with 4′6-diadomidino-2-phenylindole (DAPI). Cell images were obtained using a Leica DM5000B microscope (Leica Microsystems, Wetzlar, Germany) with filters for DAPI and fluorescein and Leica Qwin Software (Leica Microsystems). For further analysis of cyclin B1 and phospho-Aurora Kinase A/B/C, Cell Profiler image analysis software (Carpenter Lab, Cambridge, MA, USA) was used.[21] For cyclin B1 and γ-H2AX analysis, the 'IdentifyPrimaryObjects' module has been run on the DAPI image to identify the cell nuclei and 'MeasureObjectSizeShape' to determine the nucleus diameter. This was followed by the 'MeasureObjectIntensity' to measure the antibody intensity inside the nuclei. The intensity in each nucleus was normalized to the fluorescence background intensity measured in a cell-free area of the image. Nuclei were considered positive if the intensity was higher than the average intensity plus two times standard deviation of the negative control. Phospho-Aurora Kinase A/B/C was analyzed using the 'IdentifyPrimaryObjects' and 'MeasureObjectSizeShape' module. Mitosis and mitotic catastrophes were counted manually.

Western blot

Cells treated with CDV or PBS were lysed by incubation with RIPA buffer (Cell Signaling, Danvers, MA, USA) containing Protease/Phosphatase Inhibitor Cocktail for 5 min on ice, followed by brief sonication. After centrifugation, the pellet was discarded and the protein extracts were quantified using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) as per manufacturers' instructions. Equal amounts of the

extracts (10 µg for UM-SCC-47 and 93-VU-147T versus 30 µg for UPCI-SCC-72 and NOK) were separated on 8–12% SDS-PAGE and transferred to nitrocellulose membranes according to the manufacturers' instructions using Mini-Protean Tetra System (Bio-Rad, Hercules, CA, USA). Membranes were blocked with non-fat dry milk (NFDM) and incubated with primary antibodies diluted in blocking buffer (5% NFDM or BSA diluted in TBS). For detection, secondary antibodies labeled with Horseradish Peroxidase (HRP) (Dako Agilent, Santa Clara, CA, USA) and Cell signaling) were incubated on membranes during 1 h at RT. Bands were visualized with enhanced chemiluminescence (SuperSignal West Dura Extended Duration Substrate, Thermo Scientific) on the Image reader LAS-3000 (Fuji Film, Minato, Japan).

P53 mutation analysis

DNA was extracted using Maxwell FFPE LEV Automated DNA Extraction Kit (Promega Corporation, Madison, WI, USA). DNA concentration was measured using the QuantiFluor dsDNA Dye System (Promega Corporation).[22] DNA was examined using single molecule molecular inversion probes (smMIP) analysis, as previously described.[23] A smMIP-based library preparation was used to target coding sequences of the TP53 gene; NN_000546 exon 2-11.

Statistical analysis

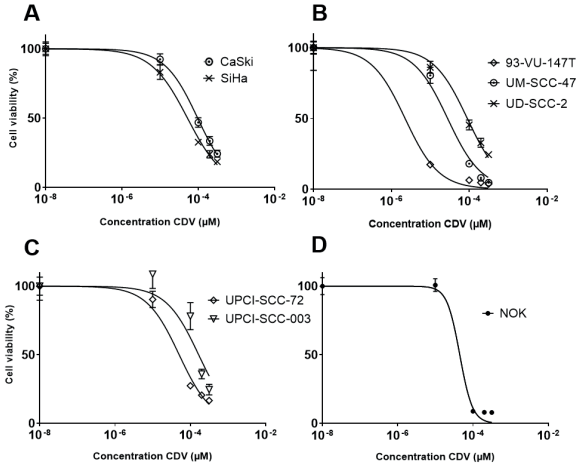
GraphPad Prism (version 6, San Diego, CA, USA) was used to conduct all statistical analyses. All results were expressed as the mean ± standard error of the mean. Independent experiments were analyzed by an unpaired Student's t-test. Levels of $p<0.05$ were considered to be of statistical significance.

Results

Effect of CDV treatment on the cell viability of HNSCC and uterine cervical carcinoma (UCC) cell lines

To determine the cell viability in the presence of CDV, all cell lines were cultured for 3, 6, and 9 days with increasing concentrations of CDV. CDV inhibited cell growth in the HPV-positive and -negative HNSCC-, the HPV-positive UCC- and the NOK cell lines as determined by the MTT assay. The anti-proliferative activity of CDV increased over time from day 3 to day 9 in all the cell lines tested. There was only a significant difference between the IC_{50} of the HPV-positive HNSCC and UCC cell lines versus the HPV-negative HNSCC cell lines after 6 days of treatment ($p=0.0102$). The IC_{50} values of day 6 and 9

varied considerably between the different cell lines (Figure 7.1). We used the IC_{50} of day 9 for further experiments.



	IC_{50} value day 6 (µM)	IC_{50} value day 9 (µM)
CaSki	139	96
SiHa	180	52
93-VU-147T	48	2
UM-SCC-47	117	30
UD-SCC-2	283	83
UPCI-SCC-72	486	54
UPCI-SCC-003	986	168
NOK	49	45

Figure 7.1 Effect of CDV on cell viability. The viability of the used cell lines was assessed using an MTT assay. The IC_{50} value is the drug dose that causes 50% growth inhibition. Showing the results of 9 days CDV treatment: (A) HPV-positive UCC cell lines, (B) HPV-positive HNSCC cell lines, (C) HPV-negative HNSCC cell lines, (D) NOK cell line, (E) Overview of IC_{50} values after 6 and 9 days of treatment. The experiments were performed in triplicate.

CDV treatment results in DNA damage

The HPV-positive cell lines 93-VU-147T and UM-SCC-47, HPV-negative cell line UPCI-SCC-72 and NOK were used to investigate DNA damage induction by CDV. The occurrence of DNA damage induction in the cell lines was confirmed by irradiation of 93-VU-147T, as there was an increase of γ-H2AX in the irradiated cells compared to the non-irradiated cells after both 4 and 24 h (Figure S7.1). All four cell lines were treated for 3 and 6 days with CDV and processed for γ-H2AX immunofluorescence. Figure 7.2A illustrates representative nuclei of the untreated and treated cells of 93-VU-147T. γ-H2AX was visible after 3 days of CDV treatment and increased further after 6 days (Figure 7.2B). The increased expression of phospho-H2AX (p-H2AX) in CDV treated cells was also seen in western blot analyses (Figure 7.2C). Similar results were observed for UM-SCC-47 and UPCI-SCC-72. NOK showed in the control and treated cells accumulation of DNA damage. There was more upregulation of γ-H2AX in the cell lines with the highest anti-proliferative effects (93-VU-147T and UM-SCC-47), compared to the cell line with the lowest anti-proliferative effect (UPCI-SCC-72).

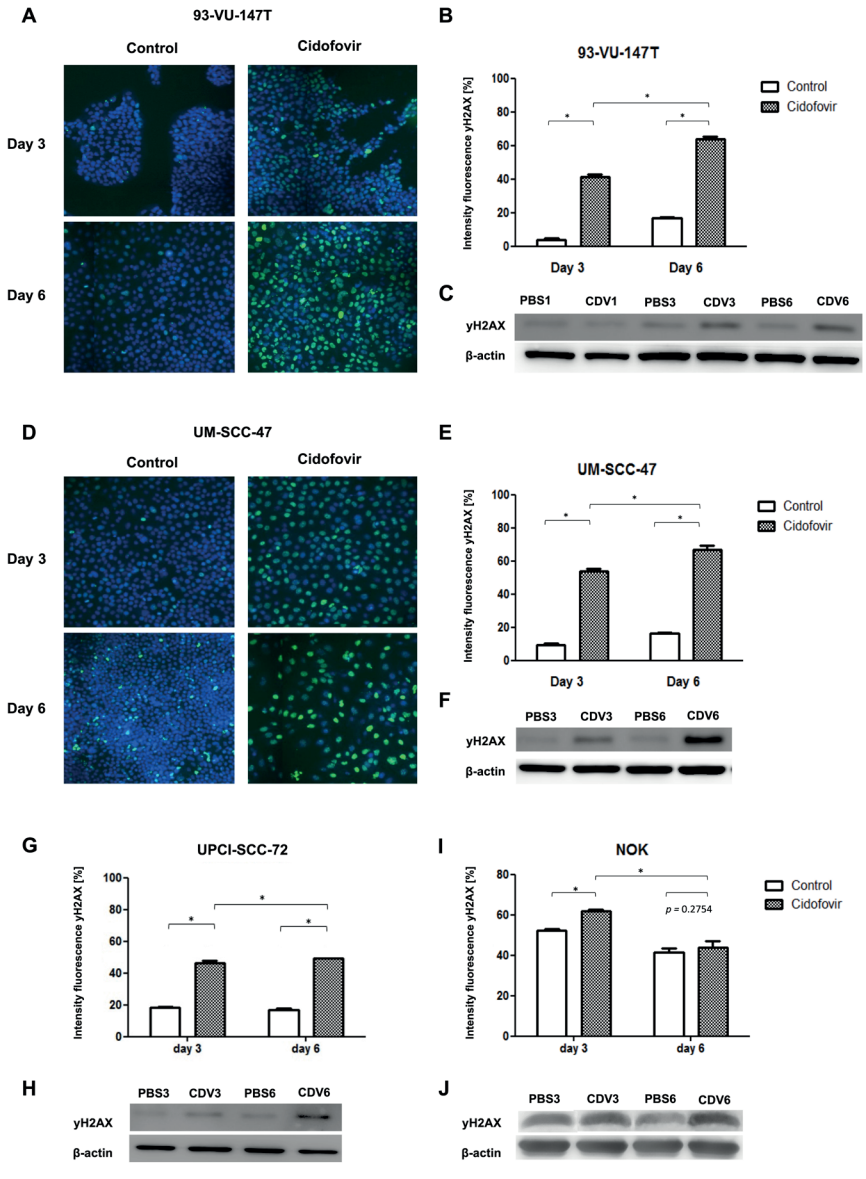


Figure 7.2 DNA damage induced by CDV as detected by γ-H2AX analysis. Cells were treated with CDV or PBS (control) and after 3 and 6 days immunostaining of γ-H2AX was performed. (A) DNA-damage is accumulated in the treated 93-VU-147T cells. Nuclei are stained with Hoechst in blue, DSBs are shown by γ-H2AX in green. (B) Quantification of γ-H2AX positive cells after 3 and 6 days CDV treatment. (C) Cell lysates of 93-VU-147T were examined by western blotting with p-H2AX after 3 and 6 days. β-actin was used as loading control. (D) DNA damage is accumulated in treated UM-SCC-47 cells. (E,F) Quantification of γ-H2AX positive cells after 3 and 6 days CDV treatment and western blotting analysis of p-H2AX for UM-SCC-47, (G,H) UPCI-SCC-72, (I,J) and NOK. Statistical significance was indicated as follows: $p<0.05$ (*). The experiments were performed in triplicate.

Activation of DNA damage response by CDV

Since increased γ-H2AX expression upon CDV treatment suggests accumulation of DNA double strand breaks (DNA DSBs), the DNA damage response pathway was investigated at protein level. In response to DNA damage, cells normally activate the DNA damage response pathway, which causes G1/S arrest via the p53 pathway and G2/M arrest via checkpoint kinases Chk1 and Chk2. We performed both western blotting of DNA damage response proteins and p53 mutation analysis on the cell lines. In 93-VU-147T, starting from day 3 a strongly increased expression of the phosphorylated checkpoint kinases Chk1 (p-Chk1) and Chk2 (p-Chk2), phosphorylated BRCA1 (p-BRCA1) and a moderately increased expression of phosphorylated p53 at ser15 (ser15p53) was observed upon CDV treatment compared to the control. In addition, cdc2 was phosphorylated at Tyr15 (p-cdc2), which is one of the two inhibition sites for the activation of the cdc2-cyclin B complex. P53 and p21 were upregulated in the treated and untreated cells (Figure 7.3A). This may be explained by presence of both wild type and mutant TP53 (L275R; allelic frequency (AF) 51%) in this cell line. In UM-SCC-47 the upregulation of the pathway appeared at day 6. In this cell line, there is only an upregulation of p53 and p21 in the CDV treated cells (Figure 7.3B). This cell line proved to harbor wild type TP53, which is down regulated by HPV oncoprotein E6. In the two HPV-positive cell lines, there was still a significant amount of DNA damage visible in the treated cells after 6 days. Analysis of UPCI-SCC-72 and NOK showed lower expression levels of the DNA damage response proteins in comparison to UM-SCC-47 and 93-VU-147T. UPCI-SCC-72 showed an upregulation of p-Chk1, p-Chk2, and ser15p53 after 6 days. p53, p-BRCA1, and p-cdc2 were detected at similar levels in the treated and untreated cells, and p21 showed lower expression levels in CDV treated cells (Figure 7.3C). This cell line harbors a pathogenic TP53 mutation (H179N; AF 100%), which is in agreement with earlier observations.[24] NOK showed upregulation of p-Chk1, p-Chk2, ser15p53, and p-cdc2. p53 and p-BRCA1 were detected at similar levels in the treated and untreated cells, and p21 showed reduced expression in CDV treated cells (Figure 5.3D). This cell line has both wild type and mutant TP53 (R213Ter; AF 39%).

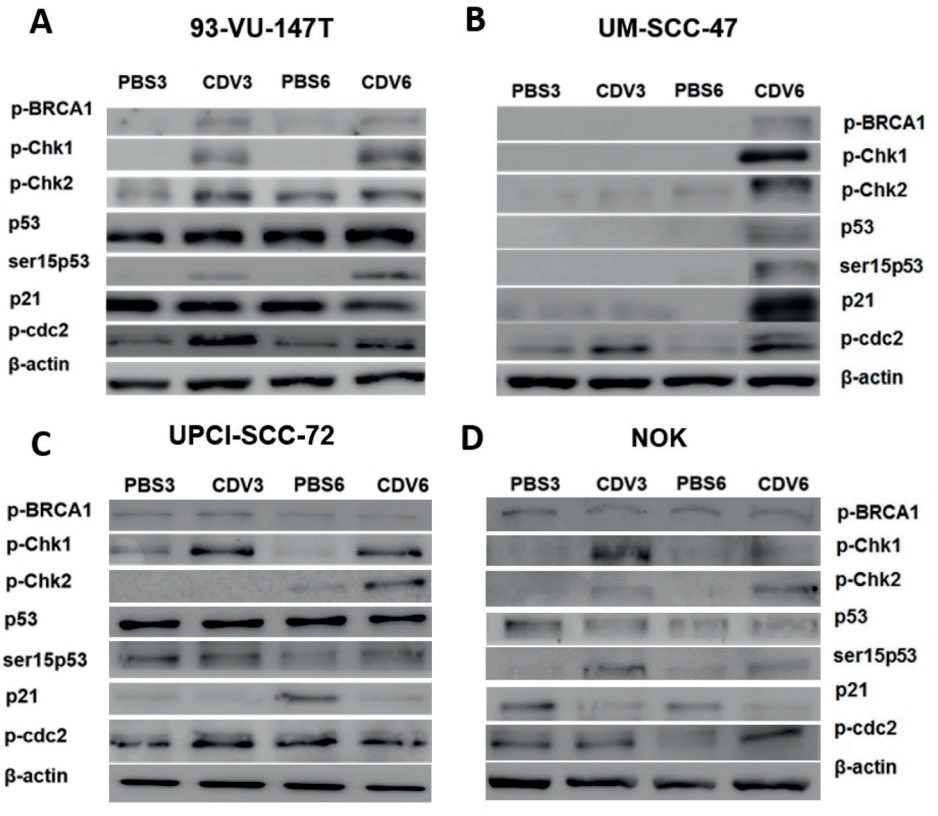


Figure 7.3 Expression levels of proteins involved in the DNA damage response pathway by western blot analysis of whole protein extracts. The cells were treated for 3 and 6 days with the IC_{50} value of CDV or control (PBS). β-actin was used as loading control. For the cell lines (A) 93-VU-147T and (B) UM-SCC-47 protein extracts of 10 µg were used, where for (C) UPCI-SCC-72 and (D) NOK protein extracts of 30µg were used. The experiments were performed in triplicate.

CDV treatment results in mitotic catastrophe

A consequence of the activation of the DNA damage response pathway may be cell cycle arrest followed by apoptosis. For this purpose, we first analyzed the cell cycle distribution by Flow Cytometry analysis after 3 and 6 days of CDV treatment. In the four cell lines there was a decrease of cells in the G1 phase and an increase of cells in the S-phase compared to the control. Furthermore, in the UM-SCC-47, UPCI-SCC-72, and NOK also after 6 days an increase in cells in the G2/M phase was observed. These results indicate that under CDV treatment cells accumulate in S -and G2/M-phase (Figure 7.4).

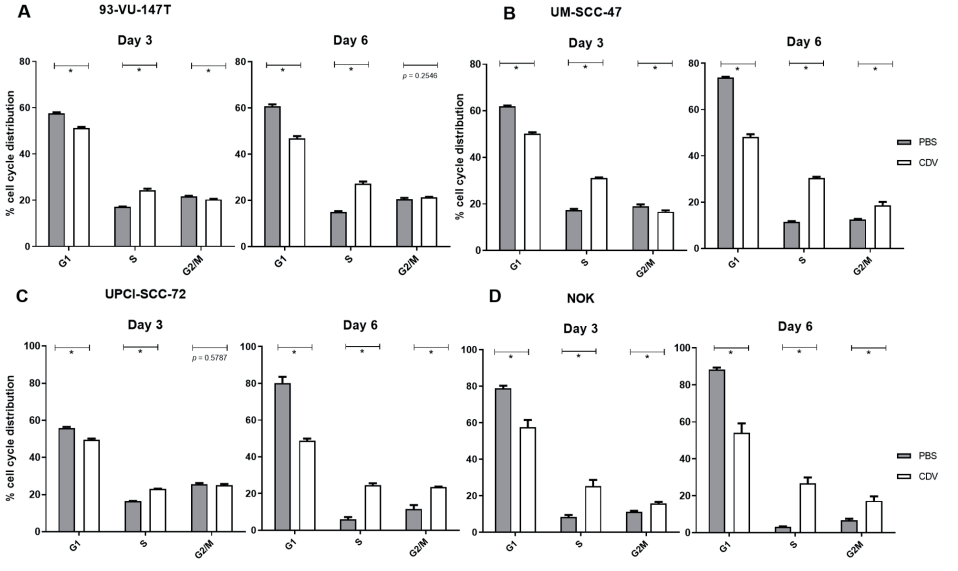


Figure 7.4 Cell cycle distribution of the HNSCC cell lines and NOK treated for 3 and 6 days with CDV or not treated (PBS). (A) 93-VU-147T, (B) UM-SCC-47, (C) UPCI-SCC-72, (D) NOK. Statistical significance was indicated as follows: $p<0.05$ (*). The experiments were performed in triplicate.

This was further confirmed by cyclin B1 immunostaining in CDV treated cell lines, showing an increase in intensity as well as the number of cyclin B1 positive cells after 6 days of CDV treatment (Figure 7.5). The most significant increase of cells in the G2/M phase after 6 days was seen for UM-SCC-47 and NOK. These cell lines showed also the most significant increase in cyclin B1 intensity after 6 days treatment.

In order to assess if cells go into apoptosis under CDV treatment, we performed an Annexin-V assay. First, all cell lines were treated with 1 µM staurosporine for 1 day, a known inducer of apoptosis. In the three HNSCC cell lines there was a strong increase of apoptotic cells observed, whereas only a slight increase was observed in the NOK cell line. In contrast, after CDV treatment there was no increase in apoptotic cells observed in the HNSCC cell lines, except for the 93-VU-147T, showing a significant increase of apoptotic cells after CDV treatment, but this was an increase of 2.7%. The NOK cell line showed a strong increase in apoptotic cells. Taken together, CDV induced apoptosis in the NOK cell line, but not in the HNSCC cell lines (Figure 7.6).

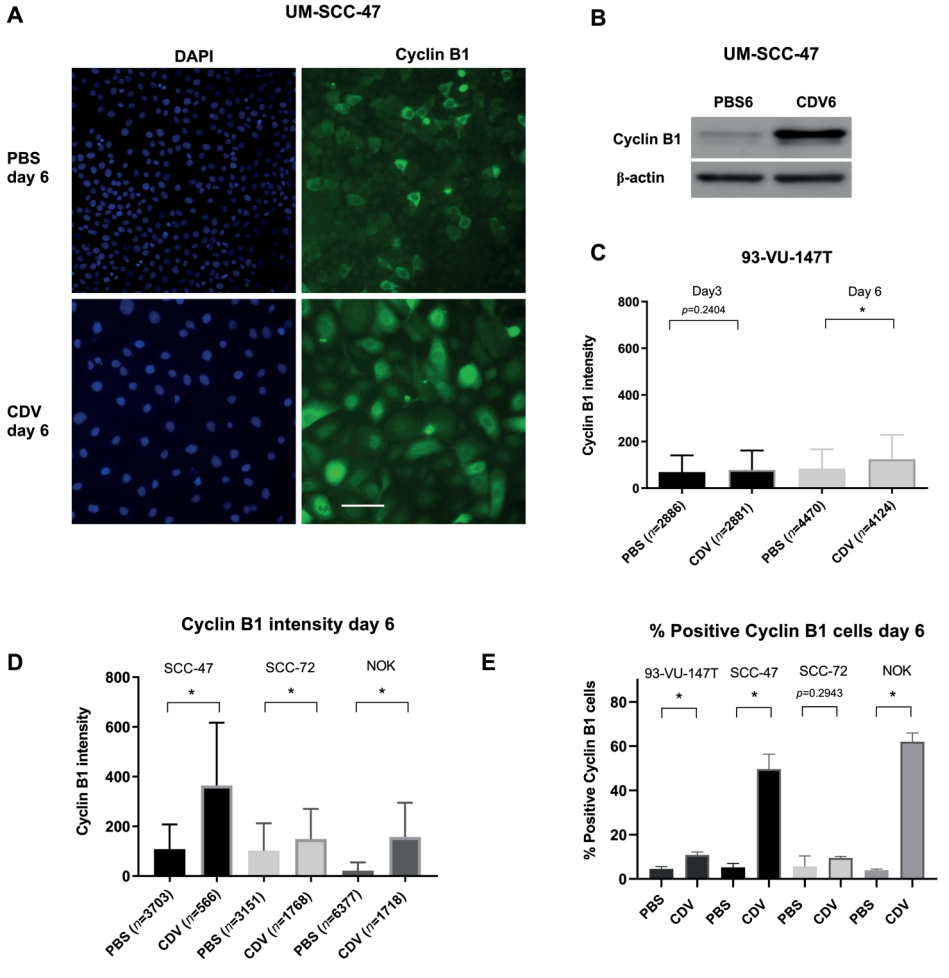


Figure 7.5 Upregulation of cyclin B1 expression in the nucleus after treatment of cell lines with CDV. The cells were treated for 3 and 6 days with the IC_{50} value of CDV followed by cyclin B1 immunofluorescence staining. Nuclei were considered positive if the intensity was higher than the average intensity plus two times standard deviation of the negative control. (A) Representative images of cyclin B1 immunofluorescence (right side) of the HPV-positive UM-SCC-47 cell line after 6 days CDV treatment vs. PBS control, left side showing blue nuclear DAPI staining. (B) Cell lysates of UM-SCC-47 were examined by western blotting of cyclin B1 after 6 days. β-actin was used as loading control. (C) cyclin B1 intensity of 93-VU-147T after 3 and 6 days of treatment. (D) cyclin B1 intensity of UM-SCC-47, UPCI-SCC-72, and NOK after 6 days of treatment. (E) % positive cyclin B1 cells of 93-VU-147T, UM-SCC-47, UPCI-SCC-72, and NOK after 6 days treatment. *n* = number of analyzed cells. Statistical significance was indicated as follows: $p<0.05$ (*). The experiments were performed in triplicate. Scale bar of (A): 100 µm.

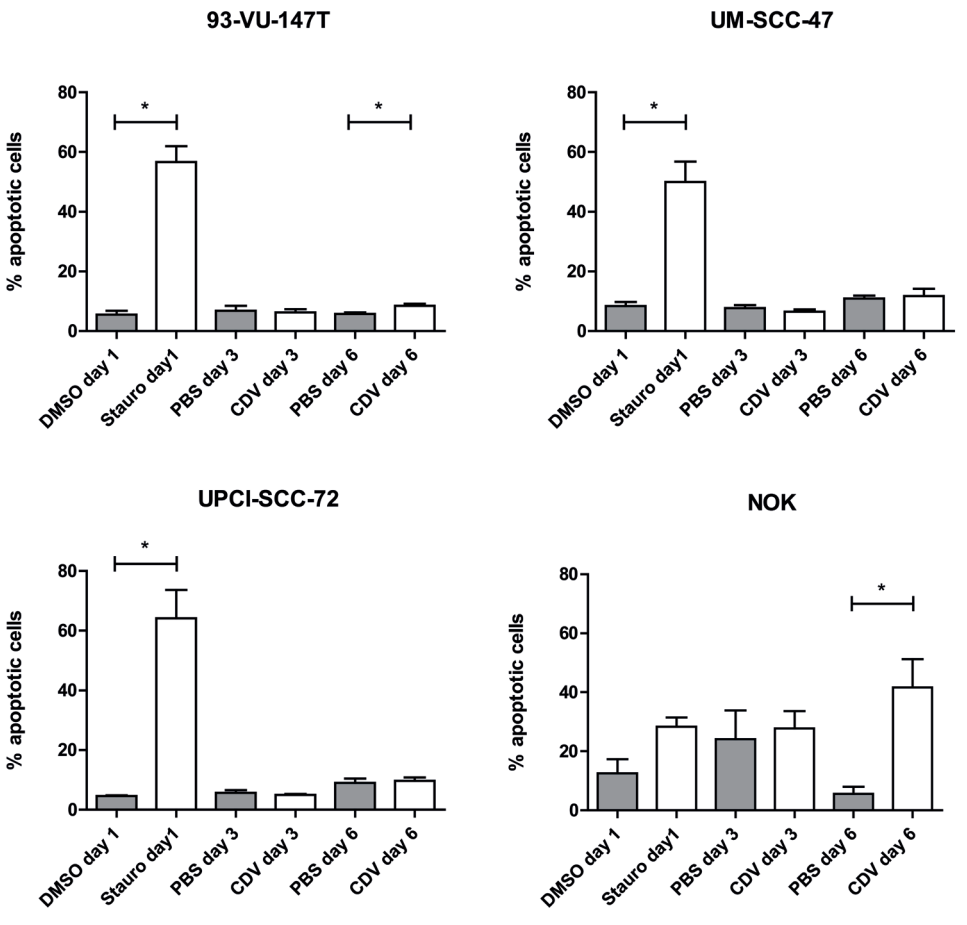


Figure 7.6 Effect of CDV treatment on induction of apoptosis. Cells were either treated for 1 day with 1µM Staurosporine, a known inducer of apoptosis or for 3 and 6 days with CDV, followed by analysis of Annexin V staining. Results are shown for (A) 93-VU-147T, (B) UM-SCC-47, (C) UPCI-SCC-72, and (D) NOK. Statistical significance was indicated as follows: $p<0.05$ (*). The experiments were performed in triplicate.

Cyclin B1 accumulation in the nucleus indicates that a part of the cells enter mitosis and with an inactive apoptosis machinery, this may lead to mitotic catastrophe. To visualize this process, we used immunofluorescence detection of phospho-Aurora Kinase, which is detected at the centrosomes along mitotic spindle microtubules and plays a role in the mitotic chromatid segregation. The first observation in these experiments was an increase in cell nuclei size after CDV treatment in comparison with the control cells (Figure S7.2). CDV treated cells showed a decrease in number of mitotic figures and an increase in cells in mitotic catastrophe (Figure 7.7).

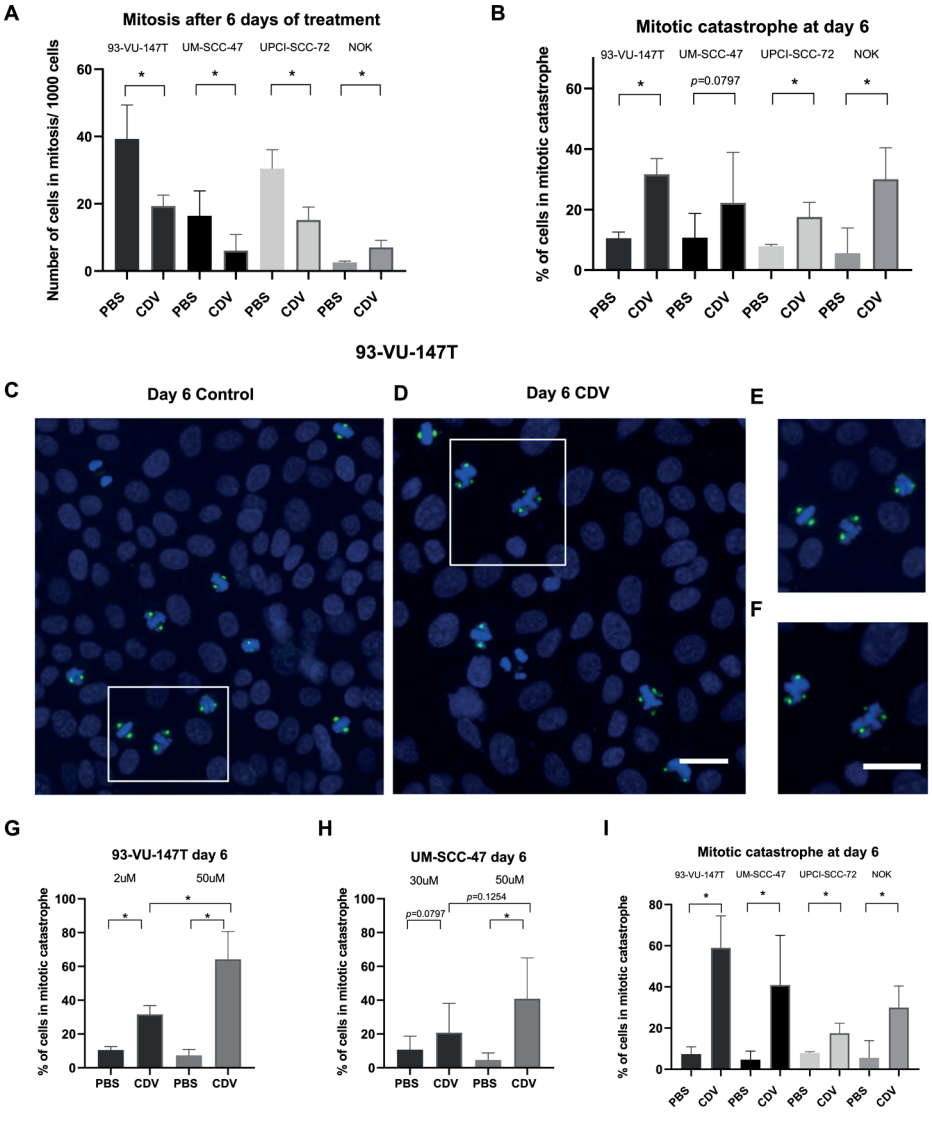


Figure 7.7 Induction of mitosis and mitotic catastrophe after treatment with CDV. The cells were treated with CDV or PBS for 3 and 6 days after which immunostaining of phospho-Aurora Kinase was performed. The cells were treated with an equal toxicity (IC_{50}) and with the same CDV concentration (50 µM). (A) The number of cells in mitosis (2 centrosomes) per 1000 counted cells and (B) percentage of cells in mitosis undergoing mitotic catastrophe when treated with PBS or CDV (IC_{50}). (C) Representative nuclei of 93-VU-147T untreated and (D) treated with CDV for 6 days. (E) Magnification of a normal mitotic figures and (F) 2 nuclei in mitotic catastrophe with multiple spindles visible (G) 93-VU-147T and (H) UM-SCC-47 cell line treated with IC_{50} vs. 50 µM. (I) Percentage of control and treated cells in mitotic catastrophe when treated with 50 µM. Statistical significance was indicated as follows: $p < 0.05$ (*). The experiments were performed in triplicate. Scale bar of (C,D,E,F): 50 µm.

NOK showed a slight increase in mitoses after treatment with CDV instead of a decrease, but also an increase in mitotic catastrophe. Because so far, the cell lines were treated with CDV concentrations resulting in equal toxicity (IC_{50} value), we also wanted to investigate if mitotic catastrophes could explain the differences in sensitivity. Indeed, Figure 7.7I shows that more mitotic catastrophes were observed with increasing sensitivity for CDV.

Discussion

The antiproliferative effects of CDV were studied in three HPV-positive, two HPV-negative HNSCC cell lines, two HPV-positive UCC cell lines and the immortalized NOK cell line. In all the cell lines the cell growth was inhibited by CDV with differences in response between the cell lines. Treatment with CDV caused DNA damage by means of DNA DSBs and as a result the DNA damage response pathway became activated. There was an accumulation of cells in the S- and G2/M phase and with an inappropriate apoptosis machinery, the cells appeared to undergo mitotic catastrophe.

CDV targets DNA viruses that encode for their own DNA polymerase. In addition, CDV has been shown to have antiproliferative properties against HPV-positive and HPV-negative malignancies in vitro and vivo.[10–12] The molecular mechanism underlying the efficacy of CDV is not completely understood, as HPV uses the host DNA polymerase for replication.[10,13] The aim of our study was to investigate the efficacy of CDV in HPV-positive and -negative HNSCC cell lines in vitro and whether this efficacy is caused by a difference in response to DNA damage. Our results show that CDV inhibits the cell growth of all the HPV-positive and -negative HNSCC, the UCC cell lines and the NOK cell line, and is more effective in the HPV-positive cell lines than in the HPV-negative cell lines after 6 days. Treatment with CDV caused DNA damage by means of DNA DSB's. There was more DNA damage visible in the two HPV-positive cell lines showing the strongest inhibition as compared to the HPV-negative cell line showing much less inhibition by CDV. The IC50 values of the cell lines SiHa, CaSki, UM-SCC-47, and UD-SCC-2 were in accordance to those found by Mertens *et al*.[25] They reported that CDV incorporation into DNA caused DNA damage, but there was no correlation between the occurrence of DNA damage and the anti-proliferative effects of CDV.

In order to further investigate the mechanism of action of CDV, we examined the activation of the DNA damage response pathway, the cell cycle and the induction of apoptosis. After treatment with CDV, the DNA damage response pathway became activated by means of phosphorylation of the DNA repair proteins (BRCA-1, Chk-1, Chk-2, and p53) in the two HPV-positive HNSCC cell lines. This effect was seen to a lesser

extent in the HPV-negative cell line and NOK cell line. In the HPV-positive cell lines only a slight upregulation of phosphorylated p53 would be expected, because of inactivation by E6, which in turn is not influenced by CDV.[14,18] This was observed in UM-SCC-47. The higher expression of p53 in 93-VU-147T might be the consequence of a TP53 mutation in one allele.

We found a S-phase arrest after 3 and 6 days CDV treatment and after 6 days there was also a G2/M arrest visible. The expression of cyclin B1 in the nucleus after treatment with CDV was also increased after 6 days. Additionally, the phosphorylation of cdc-2 on Tyr15 increased, also suggesting G2/M arrest. However, there was still a significant amount of DNA damage visible in the treated cells after 6 days, which implies that DNA repair does not occur efficiently in the HPV-positive cell lines. Similar results were found in HPV-positive UCC cells (SiHa, HeLa) by De Schutter *et al.*[14] They found that these tumor cells lacked appropriate cell cycle regulation and DNA repair as did the immortalized keratinocyte cell line (HaCaT). Earlier studies have also indicated that an impaired DNA damage repair is responsible for the elevated radiosensitivity of HPV-positive tumor cells.[26,27] An explanation for this observation might be that the expression of HPV E6 and E7 in cells hinder the homologous recombination pathway through the mislocalization of Rad51 away from the DSBs through a yet unknown mechanism.[28]

We noted that CDV treatment did not lead to an increase in Annexin-V staining. Abdulkarim *et al*. also did not detect apoptosis after CDV treatment in HPV-positive UCC and HNSCC cells and proposed cell cycle arrest to occur.[29] These results are in agreement with studies inducing DNA damage by radiotherapy in HNSCC cell lines, which also showed no occurrence of apoptosis.[26, 30]

Immunofluorescence of phospho-Aurora Kinase revealed nuclei increased in size and the presence of multiple centrosomes in CDV treated cells. Combined with the suggested G2/M arrest, this finding indicates the development of mitotic catastrophe being the predominant cause leading to cell death. Indeed, more mitotic catastrophes were observed with increasing sensitivity for CDV. Radiation as well as various antitumor drugs have been described to induce mitotic catastrophe.[31–33] Progression from G2- to M-phase is driven by the activation of the cyclin B1/cdc2 complex. Aberrant mitotic entry before the completion of DNA replication can cause mitotic catastrophe and is associated with multinuclear enlarged cells and multipolar spindles.[34] Upregulation of cyclin B1 and prolonged activation of cyclin B1/cdc2 complex are typical features of mitotic catastrophe.[35]

In contrast to the HNSCC cell lines that do not show an evident increase in apoptosis due to DNA damage caused by CDV, already substantial apoptosis was detectable at baseline in the NOK cell line which increased under CDV treatment. Assuming that NOK cells contain a least one wild-type allele of TP53, one would expect less DNA damage at

baseline and induction of apoptosis under CDV treatment because of functional p53. An alternative explanation of the observed results could be that this cell line is polyclonal, with subclones having homozygous wild-type TP53 or homozygous mutated TP53. This would explain the baseline DNA damage (in the mutated p53 cells) and detection of apoptosis under CDV treatment (occurring in the wild-type p53 cells). Hence, the question is whether or not the NOK cell line is a good normal keratinocyte control. Rather, the observed features, including the presence of a TP53 mutation, more resemble features seen in the HNSCC cell lines. The fact that normal keratinocytes cell lines that are not immortalized do not show DNA damage after CDV treatment, as has been reported by Mertens *et al.*, further underscores this suggestion.[25]

In conclusion, we found that CDV inhibits the cell growth of HPV-positive and -negative HNSCC cell lines, and was more profound in HPV-positive cell lines. CDV treated cells showed accumulation of DNA DSBs and DNA damage activation, but apoptosis did not seem to occur. Rather our data indicate the occurrence of mitotic catastrophe.

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Supplementary data

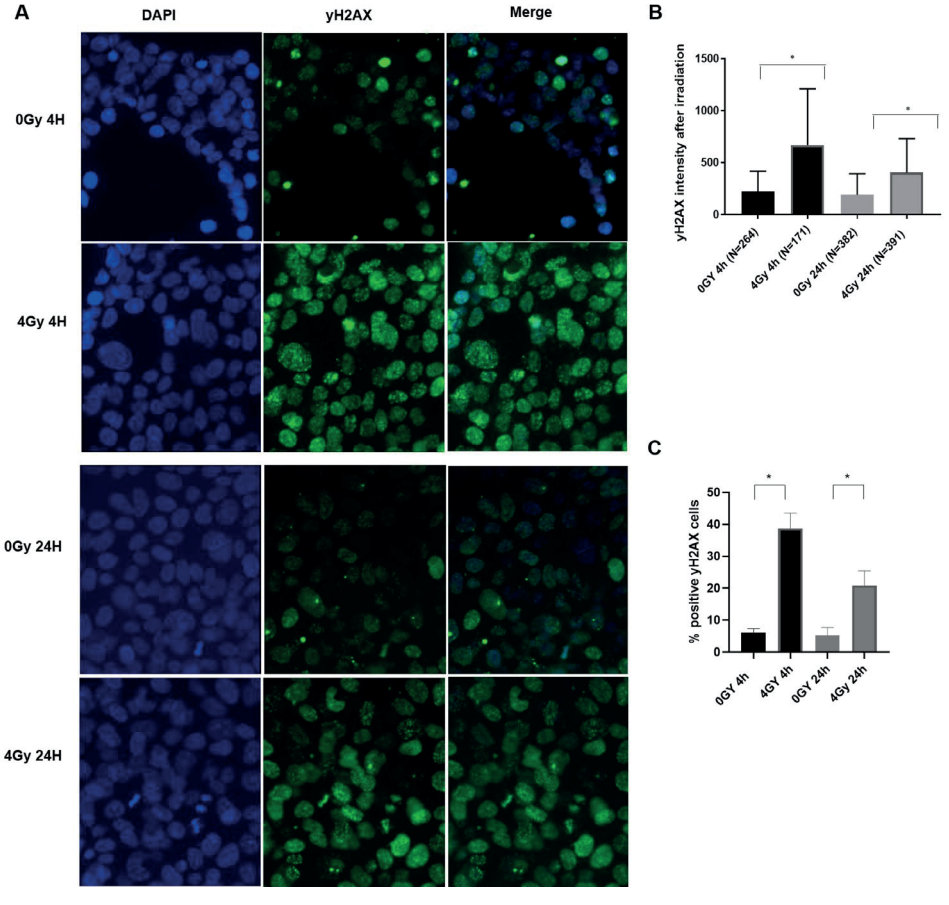

Figure S7.1 (A) The occurrence of DNA-damage in 93-VU-147T treated with 4 Gray irradiation in vitro (magnification ×200). After irradiation, the cells were cultured for 4 and 24 hours and analyzed for immunofluorescence with γ-H2AX. Nuclei are stained with DAPI in blue. DNA double strand breaks (DSBs) are shown by γ-H2AX in green. Nuclei were considered positive if the intensity was higher than the average intensity plus two times standard deviation of the negative control. (B) γ-H2AX intensity and (C) % positive γH2AX cells were quantified with the Cell Profiler image analysis program. N = number of analyzed cells. Statistical significance was indicated as follows: $p < 0.05$ (*).

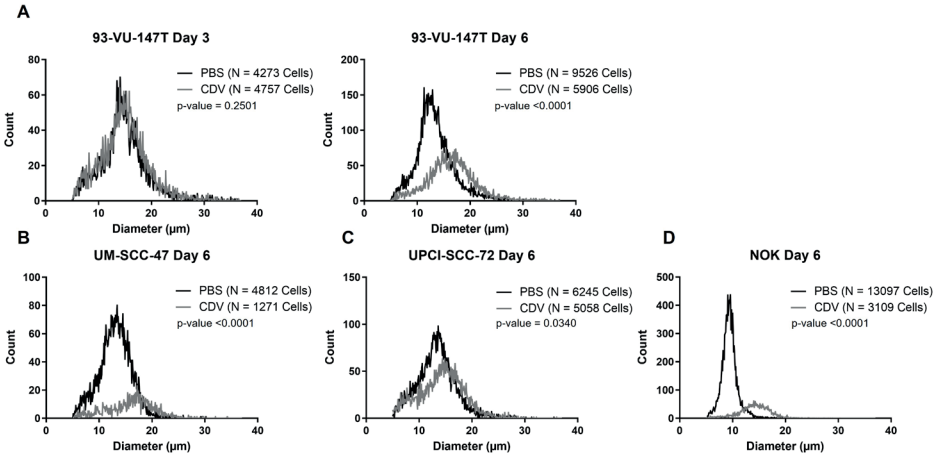

Figure S7.2 Effect of CDV treatment on the cell nucleus diameter. The cells were treated for 3 and 6 days with the IC50 value of CDV followed by immunofluorescence staining of Cyclin B1 or phospho-Aurora Kinase. After 6 days there is a significant increase in cell nucleus diameter in the different cell lines. Showing the results of (A) 93-VU-147T day 3 and 6 (B) UM-SCC-47 day 6 (C) UPCI-SCC-72 day 6 and (D) NOK day 6. N = number of cells analyzed.

Table S7.1 Primary and secondary antibodies used for Western blotting and immunofluorescence.

Primary Antibody	Size (kDa)	Dilution	Secondary Antibody	Dilution
Phospho-Histone H2A.X (Ser139). Rabbit mAb. Cell Signaling, Danvers, USA	15	1:100 (IF) 1:1000 (WB)	Anti-Rabbit IgG, HRP linked. Cell signaling	1:500 (IF) 1:1000 (WB)
Phospho-BRCA1 (Ser1524) Rabbit mAb. Cell Signaling	220	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
Phospho-Chk1 (Ser345) Rabbit mAb. Cell Signaling	56	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
Phospho-Chk2 (Thr68) Rabbit mAb. Cell Signaling	62	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
Total p53 Mouse mAb. Dako Agilent, Santa Clara, USA	53	1:1000	Polyclonal Rabbit Anti-Mouse IG/HRP. Dako Agilent	1:1000
Phospho-p53 (Ser15) Rabbit mAb. Cell Signaling	53	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
p21 Waf1/ Cip1. Rabbit mAb. Cell signaling	21	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
Phospho-cdc2 (Tyr15) Rabbit mAb. Cell Signaling	34	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
PARP (46D11) Rabbit mAb. Cell Signaling	116-89	1:1000	Anti-Rabbit IgG, HRP linked. Cell signaling	1:1000
anti-Cyclin B1 antibody. Mouse mAb. Abcam, Cambridge, UK	58	1:500 (IF) 1:1000 (WB)	Polyclonal Rabbit Anti-Mouse IG/HRP. Dako Agilent	1:500 (IF) 1:2000 (WB)
Phospho-Aurora A (Thr288)/ Aurora B (Thr232)/ Aurora C (Thr198). Rabbit mAb. Cell signalling	35,40,48	1:100	Goat anti Rabbit IgG (H + L), DyLight 488 Conjungated. Thermo Scientific	1:200
Anti-β-actin Clone AC-15. Mouse mAb. Sigma-Aldrich	42	1:2000	Polyclonal Rabbit Anti-Mouse IG/HRP. Dako Agilent	1:2000

Chapter 8

Exploring the antiproliferative effect of several PI3K/Akt/mTOR pathway and CDK4/6 inhibitors in human papillomavirus positive and negative head and neck squamous cell carcinoma cell lines

Femke Verhees*, Imke Demers*, Dion Legemaate, Robin Jacobs, Ann Hoeben, Bernd Kremer, Ernst-Jan M. Speel

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Submitted

Chapter 9

Ex vivo culture models to indicate therapy response in head and neck squamous cell carcinoma

Imke Demers, Johan Donkers, Bernd Kremer, Ernst Jan M. Speel

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is characterized by a poor 5 year survival and varying response rates to both standard-of-care and new treatments. Despite advances in medicine and treatment methods, mortality rates have hardly decreased in recent decades. Reliable patient-derived tumor models offer the chance to predict therapy response in a personalized setting, thereby improving treatment efficacy by identifying the most appropriate treatment regimen for each patient. Furthermore, ex vivo tumor models enable testing of novel therapies before introduction in clinical practice. A literature search was performed to identify relevant literature describing three-dimensional ex vivo culture models of HNSCC to examine sensitivity to chemotherapy, radiotherapy, immunotherapy and targeted therapy. We provide a comprehensive overview of the currently used three-dimensional ex vivo culture models for HNSCC with their advantages and limitations, including culture success percentage and comparison to the original tumor. Furthermore, we evaluate the potential of these models to predict patient therapy response.

Introduction

Approximately 5.5 million people worldwide suffer from a form of head and neck squamous cell carcinoma (HNSCC). The yearly incidence rate is approximately 890,000 and 450,000 people die each year as a consequence of this disease.[1,2] This makes it the seventh most prevalent cancer type in the world with a 5 year survival rate of 25–60% depending on anatomical site and stage.[3] In addition to smoking and alcohol consumption, infections with high-risk human papillomavirus (HPV) are recognized as a risk factor for the development of oropharyngeal carcinomas, specifically.[4] In recent decades, large efforts in clinical care and research have been made in order to increase the 5 year survival rate. Cisplatin-based chemotherapy was introduced more than 40 years ago and is still regarded as one of the most influential adjuvant treatments for HNSCC. However, this treatment increases the 5 year survival rate by only 4%, illustrating the limited additional value of adjuvant treatments to date.[5] More recent promising progress in the treatment of HNSCCs is the development of targeted therapies and immunotherapy. The epidermal growth factor receptor (EGFR) inhibitor cetuximab was approved by the FDA in 2006.[6] However, cetuximab was shown to be significantly more effective than standard treatment for HNSCC in only two situations—in combination with radiotherapy in patients with local progressive disease for whom chemotherapy is contraindicated, and in combination with platinum-containing chemotherapy in recurring or metastasized disease. Even though targeted therapies have given us an entirely new approach to treat HNSCCs, their impact on the 5 year survival rate is limited so far.[7,8] Much research is being conducted into specifically targeting other driver genes in oncogenic signaling pathways, such as mutations in the oncogene PIK3CA. This is achieved with the help of databases such as the Cancer Genome Atlas, which is the most comprehensive collection of integrated genomic annotations of molecular alterations in multiple cancer types.[9-11] Immunotherapy is the second new modality of treatment which has the potential to improve survival of HNSCC patients. Specifically, immune-checkpoint inhibitors are the subject of much attention. These drugs act by blocking inhibitory signals for T-cell activation, enabling tumor reactive T cells to overcome regulatory mechanisms and mount an effective anti-tumor response. At the moment, the most promising immune-checkpoint inhibitors are nivolumab and pembrolizumab, both inhibitors of the programmed cell death protein 1 (PD-1) receptor. In recurrent or metastasized platinum-resistant HNSCCs, these therapies increase the overall survival rate significantly when compared to standard treatment.[12,13] However, the overall response rate of HNSCC patients only seems to be up to 20% and the average overall survival time is increased by only a few months.[14]

Not only the development of new therapies and bringing them to market maturity, but also the increasing need to test therapies in a personalized setting form a large challenge now and in the future. At this moment, new therapies for HNSCCs are mainly tested on a group level, which means that within a group of patients, different subgroups with different therapeutic and side-effects can be included. Therefore, it is difficult to predict the therapeutic benefit of a (new) treatment for the individual patient. Furthermore, newly developed (systemic) therapies are mostly tested in patients with the most progressed, usually palliative, stages of HNSCC, whose standard therapy has failed. Often, it is not known whether the same therapy has the same (side-) effects in other cancer stages. Therefore, it is desirable that a test method becomes available in clinical practice which allows for individual testing of a certain treatment during the diagnostic work up of the patient and allows for prediction of the therapeutic effect. This would be an important improvement in personalized medicine, which is not available in clinical practice yet.

Cell culture models offer the chance to fill this gap. In an optimal setting, tumor tissue can be cultured, and different therapies can be tested to predict therapeutic outcome before treatment of the patient. Because of the progression in the development of new therapies within the past 10 years, the interest in cell culture techniques increased as well. Multiple culture models have been developed and optimized for HNSCCs, with specific attention to cultures that grow in three-dimensional (3D) architecture. Because of the growing number of cell culture models, an overview of all these models with their (dis)advantages and purposes is required. Whereas Dohmen *et al.* published a narrative review in 2015[15], there is a need for a more comprehensive update. While Dohmen *et al*. researched culture models in regard to chemotherapy (CT) or radiotherapy (RT) sensitivity testing, testing response to immunotherapies (IT) and targeted therapies (TT) has become more important since. Therefore, we review current literature on HNSCC primary 3D culture models and their application as preclinical predication assays for therapy response.

Materials and methods

A systematic literature search was performed using the PubMed and EMBASE databases. The search was built to include all articles discussing primary HNSCC 3D culturing techniques on which CT, RT, IT, and/or TT was tested (Supplementary File S9.1). These articles were first screened based on title and abstract, after which a full-text screen of the selected articles was performed. Studies were included if they described a 3D

culture technique of fresh primary HNSCC tissue in combination with sensitivity testing to aforementioned therapies. Studies describing culture models involving animals were excluded. Conference abstracts and reviews were also excluded, but their references were screened for additional articles. The titles and abstracts of the references of all included articles were screened as well (Figure 9.1).

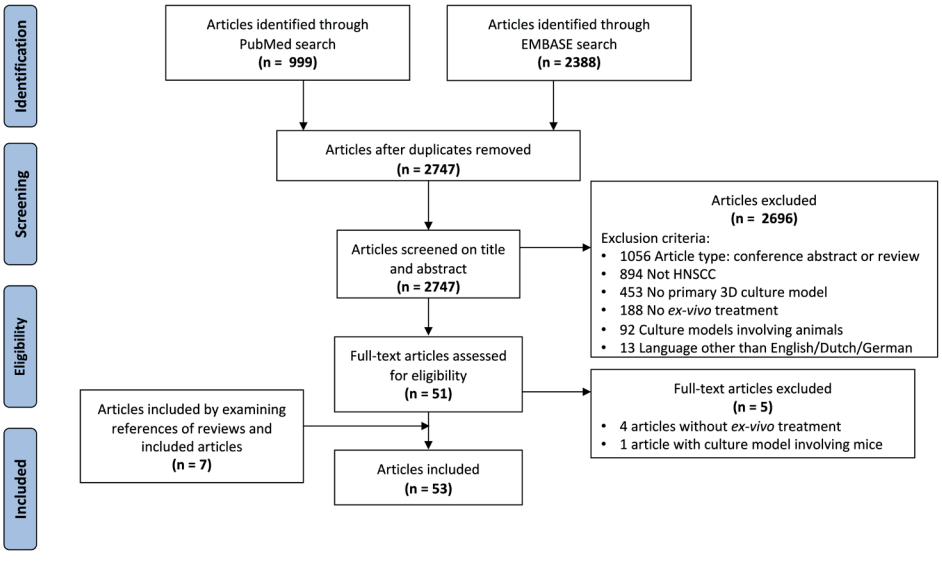

Figure 9.1 Flow diagram of the systematic literature search performed.

Full tables with all 53 included articles and their extracted data can be found in the Supplementary Materials (Tables S9.1 and S9.2). Of these, key publications were selected and presented in the results section. This selection was performed by two authors scoring all articles based on predefined criteria (Table 9.1). All articles scoring 5 or more points were defined as key publications. Studies describing a correlation between ex vivo and patient treatment response were selected and of all culture models at least the highest scoring article is presented since not every culture model was described by an article scoring at least 5 points. The screening of articles by titles and abstracts was performed by one author, whereas the final selection, data extraction and scoring of articles based on the full texts was performed by two authors independently. Final selection was based on the consensus of all authors.

Table 9.1 Criteria and scoring for the selection of key publications.

Criterium	Scoring
Reproducibility of methods	0, 1, or 2 points
Number of patients included	0–9: 0 points, 10–29: 0.5 points, ≥30: 1 point
Success percentage	Not reported: 0 points, reported: 1 point
Culture duration	Not reported: 0 points, reported: 1 point
Complete results on culture quality and treatment response	0, 1, or 2 points

Results

Overview of ex vivo culture models used for HNSCC

Based upon the articles found in the systematic search and additional relevant literature, an overview of the most commonly used primary culture models for HNSCCs was composed. In this overview, we aim to give a description, nomenclature and (dis)advantages of each model (Table 9.2). For the remainder of this review, this terminology will be used to describe the culture models in the included studies. To make the overview as complete as possible, 2D monolayer and patient-derived xenograft (PDX) culture models were also included for comparison purposes, even though these were excluded in the systematic search.

Adherent monolayer

In order to establish a 2D monolayer culture, the tumor sample is dissociated into single cells and cultured at the bottom of a container, such as a culture flask or Petri dish (Figures 9.2 and 9.3A). Due to clonal expansion, the cells will cover the entire surface. Synthetic culture medium, often supplemented with fetal bovine serum and L-glutamine, is used to provide cells with nutrients and growth factors. Cell culturing is usually performed at body temperature (37°C) and a subculture is achieved by detaching the cells from the plastic surface with trypsin and/or EDTA when the cells reach confluency. Since the technique is relatively inexpensive, well established and relatively easy to perform, it remains one of the most used culture techniques in the world.[44] However, when culturing cells as a monolayer, the original tissue preservation is lost. This causes changes in cell morphology and interactions. In addition, as monolayer cultures are usually formed due to clonal expansion, selection for one cell type often takes place. This results in a monoclonal culture that may also change phenotypically and genotypically over time.[45,46] This monoclonality is in contrast to the original tissue containing multiple cell types. These discrepancies between monolayer cultures and the *in vivo* situation have caused the scientific community to start developing 3D culture techniques, which eliminate some of these shortcomings.

Table 9.2 Overview of currently used primary culture models for HNSCC.

Culture Model	Description	Examples	Advantages [a]	Disadvantages [a]	Ref
2D					
Adherent monolayer	Cells grown as a monolayer attached to a plastic surface	• Cell Adhesive Matrix assay • FLAVINO assay	• Appropriate for most cell types • Access to nutrients and oxygen is no limiting factor • Standardized protocols • Simplicity • Low cost • High reproducibility	• Different cell morphology • No natural structure of tumor • Limited cell–cell and cell–ECM interactions • No gradient in nutrient and oxygen availability as in vivo • Not all cell suspensions will grow in monolayer setting	16-19
3D					
Multicellular spheroids	Cell aggregates grown from either single-cell cultures or tissue fragments with multiple cell types	• Suspension culture • Fragment spheroids • Scaffold based • Agitation based * • Hanging-drop culture • Magnetic levitation *	• Physiological cell–cell and cell–ECM interactions • Multiple cell types resembling in vivo situation • Gradients of nutrient and oxygen availability • High reproducibility • Suitable for HTS	• Often not uniform in size • Simplified architecture • Difficult to maintain long term • Lack potency for self-renewal and differentiation • Possibility of central necrosis	20-23
CSC-enriched spheroids	Spheroids enriched for CSCs or cells with stem cell traits, formed by clonal proliferation.	• Suspension culture • Hanging-drop culture	• Suitable to study CSC-related characteristics • Potential for self-renewal and differentiation	• Absence of non-tumor cells • No histological preservation of original tumor • Identification of CSCs from solid tumors remains evasive • Possibility of central necrosis	24-27
Organoids	Collection of cell types that develops from stem cells or progenitors and self-organizes in a manner similar to in vivo	• Embedded in matrix • Air–liquid interface • CTOS method	• Physiological cell–cell and cell–ECM interactions • Gradients of nutrient and oxygen availability • Composition and architecture resembling primary tissue • Capacity of self-renewal and differentiation • Can be cryopreserved and expanded	• Often not uniform in size • May lack key cell types • Hard to reach in vivo maturity • Possibility of central necrosis • Time consuming • High costs for media and growth factors • Less suitable for HTS	28-31

Table 9.2 (continued)

Culture Model	Description	Examples	Advantages [a]	Disadvantages [a]	Ref
3D					
Histocultures	Tumor tissue left intact by only mechanical cutting/slicing	• Histoculture Drug Response Assay • Tumor slices • Tumor fragments	• Tumor environment as in vivo • Maintains tumor heterogeneity, including stromal/immune cells • No tissue pre-processing	• Relatively much tumor tissue needed for establishment • Difficult to maintain long term • Not suitable for HTS	32-37
Patient-derived xenograft (PDX)	Patient-derived cancer cells are injected into immune-deficient mice	• Subcutaneous implantation • Orthotopic implantation	• Maintains tumor microenvironment and heterogeneity • Captures complexity of metastatic processes in a living system • Intact endocrine system	• Mice have deficient immunity • Differences in microenvironment between mice and human • Time consuming • High costs • Ethical issues of animal use	38-40
Microdevices	System that provides a precisely controlled culture environment	• Microfluidic systems • Tumor-on-a-chip models	• Can be combined with any culture technique • Allows continuous perfusion with culture medium • Tightly controlled culture conditions	• Requires external materials (tubes, pumps, connectors) to operate • Complex to control • High costs • Still in early development	41-43

ECM=extracellular matrix, HTS=high-throughput screening, CTOS=cancer tissue-originated spheroid, CSCs=cancer stem cells, *=not described for HNSCC tissues in included literature of this review, [a]=(Dis)advantages are extracted or deduced from references and may not be all encompassing. (Dis)advantages can be different for specific methods/examples.

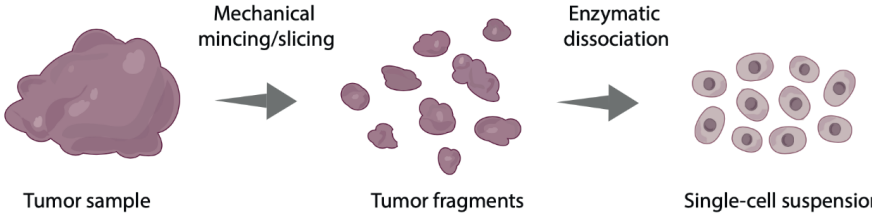

Figure 9.2 Processing of the primary tumor sample into tumor fragments by mechanical modification and subsequently into a single-cell suspension by enzymatic dissociation.

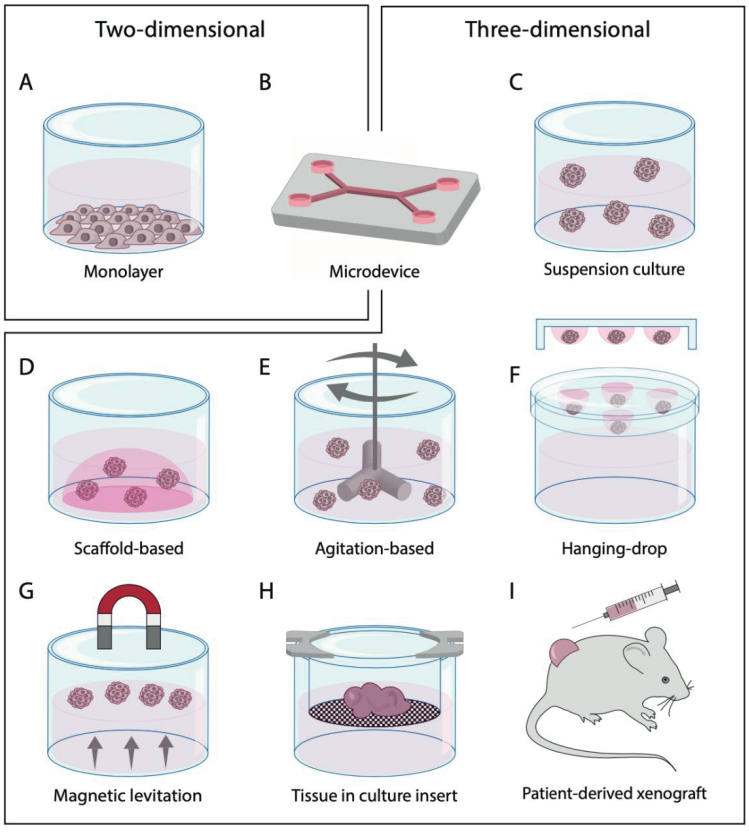

Figure 9.3 Primary cell culture techniques divided into two-dimensional and three-dimensional models. (A) 2D monolayer from single-cell suspension; (B) micro(fluidic) device; (C) spheroids in suspension culture; (D) spheroids embedded in a scaffold-based system; (E) spheroids in an agitation-based system, e.g., a spinner flask; (F) spheroids in hanging-drop cultures; (G) spheroids formed by magnetic levitation; (H) histoculture in culture insert; (I) patient-derived xenograft mouse model with subcutaneous injection. Image of xenograft was modified from Servier Medical Art (http://smart.servier.com/), licensed under a Creative Common Attribution 3.0 Unported License (https://creativecommons.org/licences/by/3.0/).

Three-dimensional culture models

Three-dimensional culture models have become more popular in recent years because they mimic the tumor architecture inside the body more accurately compared to monolayer cultures. In general, 3D models provide a more realistic way to grow tumor cells, including a better imitation of the variable access to nutrients and oxygen, enabling assessment of the tissue-penetrating ability of drugs and allowing for interaction between different cell types. All 3D culture models have their own unique advantages, disadvantages and applications. As there is a lot of confusing and overlapping terminology for the 3D culture models, especially for sphere-type models, this overview aims to clarify this nomenclature.

Multicellular spheroids

Multicellular spheroids are cell aggregates from single-cell suspensions or tissue fragments containing multiple cell types from primary tissue (Figures 9.2 and 9.4A). To achieve cell aggregation, a multitude of methods are available including suspension cultures with ultra-low attachment plates or hanging-drop cultures (Figure 9.3C–G). For this purpose, primary tissue can either be enzymatically dissociated into single cells or it can be only mechanically minced into small fragments.[22] Spheroids formed by the latter are also referred to as organotypic multicellular spheroids or fragment spheroids. These spheroids leave part of the original microenvironment of the original tissue intact as the cells are not dissociated from their original environment.[20]

Cancer stem cell-enriched spheroids

The cancer stem cell (CSC)-enriched spheroids (also referred to as tumorspheres or tumor-derived spheroids in literature) originate from CSC or cells with stem cell traits. The enrichment for these CSCs is often performed by cell sorting (i.e., based on CD44 expression) and assessment of self-renewal capability. These CSCs are grown in low-adherent conditions using stem cell medium in order to form spherical structures.[47] Whereas cell aggregation may occur by the low-adherent conditions, CSC-enriched spheroids are predominantly formed by clonal expansion of the CSCs (Figure 9.4B). The self-renewal capacity of the CSCs gives these spheroids the potential to proliferate and differentiate, which is of importance when studying stem cell characteristics and behavior. In contrast to the multicellular spheroids, CSC-enriched spheroids generally contain only one cell type (monoclonal) and therefore differ substantially from the original tumor on a histological level. It also remains a challenge to correctly identify the cells that could be denominated as CSCs, which is currently only possible by evaluation of stem cell-specific markers or self-renewal capacity.[47]

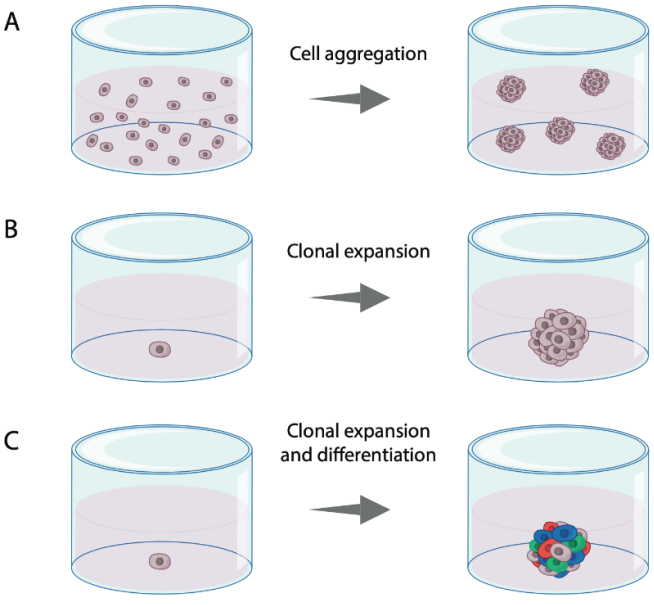

Figure 9.4 Principles of sphere formation from primary tumor cells. (A) Sphere formed by aggregation of multiple cells in a single-cell suspension; (B) sphere formed by clonal expansion of a single cell with proliferating potential; (C) sphere formed by clonal expansion and lineage-dependent differentiation of a single cell with proliferating potential.

Organoids

Already in 1992, organoids from disaggregated carcinomas were established in athymic mice by Köpf-Maier and colleagues.[28] The first organoid cultures for HNSCC without the use of animals were described in 2018.[30,48] Organoids develop from stem cells or organ progenitors but contain multiple types of organ/tumor-specific cells through lineage-dependent differentiation (Figure 9.4C). This is achieved by embedding the fresh primary dissociated tumor cells in an extracellular matrix (ECM), such as Matrigel, and providing the cultures with specifically supplemented growth medium (Figure 9.3D). This causes the organoid to self-organize in a manner similar to the in vivo situation. Because the organoid culture technique is relatively new and generally complex, it still faces practical challenges, including the fact that it is time consuming, high costs and the need for well-established protocols which differ depending on the tumor type.

Histocultures

Histocultures consist of tissue that has only been modified mechanically without enzymatic dissociation (Figure 9.2). This culture model is described in the literature as

tumor slices, tumor fragments, tumor particles, (ex vivo) tumor explants or tumor sections. Histocultures preserve the tumor cells in their original microenvironment, including the ECM and immune, stromal and vascular cells. The tumor cultures are generally cultured at air–medium interface, using, e.g., culture inserts (Figure 9.3H). The major challenge of culturing histocultures is the quick deterioration of the tissue and loss of cell viability.[36] This makes them not suitable for culturing for extended periods of time at this moment. Tissue viability might be prolonged by the use of a supportive matrix.[49] Nevertheless, the histoculture model is hampered by a relatively short viable period of 3–6 days and other disadvantages such as low throughput and low reproducibility.

Patient-Derived Xenograft (PDX)

A different way of growing 3D tumor models is to implant the patient-derived tumor cells or tumor fragments into immune-deficient mice (Figure 9.3I). This allows outgrowth of the tumor cells and thereby retaining the intratumor heterogeneity.[50] Evaluation of tumor growth within mice offers the chance to evaluate tumor formation in a living system, which allows investigation of metastatic processes and the influence of the endocrine system. Nevertheless, the culture technique does have major flaws. The most fundamental flaw is that the tumor and its microenvironment are slowly mingled with ECM and cells from the mouse, which will likely influence the test results. In addition, the PDX cultures usually have a long generation time (approximately 2–12 months) and are fairly expensive due to maintenance of animals and their facilities.[51] In addition, PDX cultures are unsuited to assess the role of the immune system in relation to therapy response due to the immune-deficient nature of the mice. Lastly, ethical issues are involved with the use of animals in (cancer) research. These include using the minimum number of animals required, allowing precise statistical analysis and results, and preventing the repetition of experiments. Another ethical concern is the physical and moral well-being of the animals, for which efforts should be undertaken to replace, reduce, and refine animal experiments (three Rs principle).[52,53] For these reasons, we decided not to evaluate PDX models as a potential preclinical model in the current review.

Microdevices

Microdevices are unique as they can be used to culture multiple monolayer and 3D models (Figure 9.3B).[54] Therefore, overlap may be observed between microdevice cultures and the aforementioned culture models, e.g., regarding culture success rate. Microdevices allow a controlled culture-environment, including continuous perfusion of the culture with medium, mimicking constant blood flow in the in vivo situation. In addition, a microdevice can offer special structures to control the position, shape,

function and both chemical and physical cell environments.[55] The main drawback of using a microdevice in the culture setup is that all these factors increase the complexity substantially.[56] Besides that, costs are still high for the setup of a microdevice system and read-out methods are limited.

Characteristics of primary 3D culture models of HNSCC and suitability for drug response testing

Key publications describing the use of primary 3D cultures of HNSCC for sensitivity testing to CT/RT and IT/TT are presented in Tables 9.3 and 9.4, respectively. For each study, model characteristics, technical aspects such as culture duration and success percentage, and main results are presented. Table 9.3 includes an overview of the correlation between therapy response observed ex vivo compared to the clinical response of the patient with predictive values including sensitivity and specificity. For IT/TT (Table 9.4), this correlation has not been reported in any of the reviewed articles. The culture models are grouped by technique in chronological order. Full tables with all examined articles can be found in the Supplementary Materials (Tables S9.1 and S9.2). Below, studies using primary culture models for drug sensitivity testing are discussed per culture model.

Multicellular spheroids

Four out of seven studies using multicellular spheroids reported success percentages of 50–100%, >90% and two times 100%. The tumors originated from different HNSCC locations, including oropharynx, hypopharynx, larynx, tongue, and unknown primary site. In regard to culture success rate, one study reported that spheroid formation with primary cells obtained from biopsies was more reliable and reproducible in ultra-low attachment plates than in a hanging-drop system.[22] The range of culture duration of these spheroids was 4–21 days, with an average of 10–15 days.

CT/RT

One study used a multicellular spheroid model of HNSCC for cisplatin, 5-FU, and radiotherapy sensitivity testing.[57] This study analyzed aldehyde dehydrogenase (ALD)-positive and ALD-negative subpopulations in these spheroids and examined ALD activity compared to primary monolayer cell cultures. Spheroid cultures show 1–2% apoptosis after treatment, in comparison with 5–25% in 2D monolayer cultures. This observation indicates differences in response to drugs between 2D and 3D culture models and suggests that the 3D architecture might be a better representation of the tumor in vivo.

Table 9.3 Overview of selected studies using chemotherapy or radiotherapy on various cultures from HNSCC tissue.

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment	In-Patient Treatment	Correlation Ex Vivo vs. In Patient
Leong, 2014[57]	Multicellular Spheroids	3	4–9	-	Cisplatin, 5-FU, Etoposide, RT	FACS	-	Spheroids were more resistant to all treatments than monolayers. Cells with a high ALD expression were resistant to cytotoxic agents.	-	-
Lim, 2011[24]	CSC-enriched Spheroids	47	14	6%	Cisplatin, 5-FU, Paclitaxel, Docetaxel	MTT	-	Undifferentiated spheroid cells were significantly more resistant to chemotherapeutic agents than differentiated spheroid cells.	-	-
Tanaka, 2018[30]	Organoids	43	8–30	30.2%	Cisplatin, Docetaxel	Relative organoid area day 1 vs. day 8	Histological patterns, vimentin expression and CD44/ALDH1A1 ratios were similar between organoids and the original tumor.	Cisplatin IC50: 0.92–1.02 µM Docetaxel IC50: 1.46–3.75 nM	-	-
Driehuis, 2019[31]	Organoids	34	42	60%	Cisplatin, Carboplatin, RT	CellTiter-Glo 3-D Assay	Tumor-specific histopathological changes were retained in culture. Organoids contain only transformed tumor cells.	IC50 cisplatin: 0.5–12.8 µM IC50 carboplatin: 3.0–81.9 µM AUC RT: 238–698	RT	6/7 matched response: 3 positive outcomes with sensitive organoid, 3 no response with non-sensitive organoid
Au, 1993[58]	Histocultures	83	9	59%	Cisplatin, 5-FU, MMC	^{3}H-TdR	Most histocultures contained areas of viable and necrotic tissue. Histology of viable regions of the cultures was similar to that of the fresh tumor.	Primary tumors mean IC50: 5-FU: 0.68 ± 0.74 µg/mL Cisplatin: 3.77 ± 2.42 µg/mL MMC: 0.25 ± 0.13 µg/mL 9/47 tumors not sensitive	-	-

Table 9.3 (continued)

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment	In-Patient Treatment	Correlation Ex Vivo vs. In Patient
Robbins, 1994[32]	Histocultures (HDRA)	26	3–11	88%	Cisplatin	^{3}H-TdR	-	84% reduction in the number of cells incorporating ^{3}H-TdR in drug-treated samples compared to control samples is used as the cut off for sensitivity in vitro	Cisplatin	Sensitivity: 71% Specificity: 78% PPV: 83% NPV: 64%
Robbins, 1996[59]	Histocultures (HDRA)	43	6–9	91%	Cisplatin	^{3}H-TdR	-	Sensitivity overall: 1.5µg/mL: 22% 15 µg/mL: 62% 37.5 µg/mL: 83% Factor growth inhibition Untreated lesions: ×2.44 Recurrent tumors: ×5.56	-	-
Welters, 1999[60]	Histocultures (3 mm^{3})	8	1	-	Cisplatin	32-P labeling	-	Because most of the HNSCC biopsies were too small to perform analyses at several time points, no adduct levels over time could be measured.	Cisplatin	DNA adduct levels partial responder vs. non- responder: Pt-GG: 27.4 vs. 5.1 Pt-AG: 13.2 vs. 2.4
Singh, 2002[61]	Histocultures (HDRA)	41	2	98%	Cisplatin, 5-FU	MTT	-	number of resistant tumors: 13/41 resistant to 5-FU, 13/41 resistant to cisplatin, 11/41 resistant to both	Cisplatin, 5-FU, RT	2 year CSS sensitive vs. not-sensitive: 5-FU: 85% vs. 64% Cisplatin: 86% vs. 63% 5-FU + cisplatin: 85% vs. 63%
Ariyoshi, 2003[62]	Histocultures (HDRA)	19	7	100%	Cisplatin, Docetaxel 5-FU, THP, ADM, BLM	MTT	-	Sensitivity rate per drug: Cisplatin: 78.9% Docetaxel: 100% 5-FU: 38.4% THP: 7.7% ADM: 0% BLM: 21.4%	Cisplatin, 5-FU, THP, BLM	Accuracy: 78.9% Sensitivity: 86.7% Specificity: 50% TPR: 86.7% TNR: 50%

Table 9.3 (continued)

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment	In-Patient Treatment	Correlation Ex Vivo vs. In Patient
Hasegawa, 2006[63]	Histocultures (HDRA)	49	7	100%	Cisplatin, 5-FU	MTT	-	Cisplatin efficacy rate: 36.7–71.4% 5-FU 120µg/mL vs. 300µg/mL efficacy rate: 23.1–57.7% vs. 70.8–75.0%	Cisplatin, 5-FU	Prediction rate: 77.8% Sensitivity: 90.9% Specificity: 57.1% TPR: 76.9% TNR: 80.0%
Hasegawa, 2008[64]	Histocultures (HDRA)	44	7	82%	Cisplatin, 5-FU	MTT	-	Mean I.I. 5-FU: 36.76% Mean I.I. cisplatin: 35.65% 5-FU sensitivity: 21/44 (58.3%) Cisplatin sensitivity: 21/44 (58.3%)	-	-
Pathak, 2008[65]	Histocultures (HDRA)	57	8	91%	Cisplatin, 5-FU, MTX	MTT	-	Cisplatin sensitivity: 52% 5-FU sensitivity: 46% MTX sensitivity: 52% Sensitive to one drug: 88%	Cisplatin, 5-FU, MTX, Paclitaxel, Ifosfamide	Accuracy: 74% Sensitivity: 79% Specificity: 71% PPV: 69% NPV: 80%
Gerlach, 2013[34]	Histocultures (Tissue slices 350 µm)	12	3–6	-	Cisplatin, Docetaxel	IHC	Cultures maintained morphological features and γH2AX expression for up to 6 days compared to original histopathology.	Control vs. cisplatin vs. docetaxel: # nuclei: ±400 vs. ±125 vs. ±150 % caspase-3-positive cells: ±2% vs. ±6% vs. ±22%	-	-
Suzuki, 2015[66]	Histocultures (HDRA)	28	7	100%	Cisplatin	MTT	-	SUV_{max}: 14.04 ± 7.52 I.I.: 50.98 ± 26.6 SUV_{max} was significantly correlated with the I.I. cisplatin (p<0.04, R^2=0.17)	Cisplatin, 5-FU, RT	SUV_{max} ≥ 10.5 and I.I. cisplatin < 50 were significantly correlated with shorter OS

Table 9.3 (continued)

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment	In-Patient Treatment	Correlation Ex Vivo vs. In Patient
Engelmann, 2020[49]	Histocultures	13	7–21	100%	RT	IHC	Comparable histological and morphological characteristics were observed between primary non-HPV tumors and histocultures after 14 days. Cultures display heterogeneous growth patterns on dermal equivalent.	Irradiation of tissues resulted in a slight increase or decrease in Ki-67 expression compared to control: Overall: +0.22% Non-HPV driven: −5.28% HPV driven: +3.89% 2/5 tumors showed increase in apoptotic cells after fractionated irradiation.	RT	One patient developed local relapse, with the corresponding histoculture showing an invasive growth pattern
Hattersley, 2012[41]	Microdevice	23	8	91%	Cisplatin, 5-FU	LDH and cytochrome-c release, WST-1 metabolism	The nuclei of the tissue 72 h after culture appear intact and loss of cell cohesion is minimal. There was no necrosis in the center of the biopsy.	% viable cells after treatment: Control: 72% ± 15.6 5-FU: 45% ± 22.3 Cisplatin: 44% ± 20.2 5-FU + cisplatin: 30% ± 23.7 All treatments showed a higher release of cytochrome-c than control samples. (p<0.01)	-	-
Carr, 2014[67]	Microdevice	35	2–3	-	RT	LDH and cytochrome-c release, IHC	There was no difference between the apoptotic index (AI) of the uncultured and cultured control tissue (p = 0.29).	AI 0 Gy: ±1% AI 5 Gy: ±7% AI 10 Gy: ±15% AI 20 Gy: ±20% AI 40 Gy: ±45% (p=0.006)	-	-

Table 9.3 (continued)

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment	In-Patient Treatment	Correlation Ex Vivo vs. In Patient
Cheah, 2017[68]	Microdevice	5	2	100%	Cisplatin, RT	LDH release, IHC, TUNEL	-	γH2AX: 1/5 sign. response CK18-LI: 2/5 sign. response TUNEL: 3/4 sign. response Ki-67: 0/5 sign. response	RT, CRT	Matched responses for 2/2 patients (for 2/4 markers)
Kennedy, 2019[69]	Microdevice	18	3	67%	Cisplatin, RT	IHC	The average Ki-67 index decreased in the control sample (7.9% ± 3.5) relative to the pre-culture sample. No difference in γH2AX expression and apoptosis between pre-culture and control samples.	Control vs. RT: BrdU: 13.3% vs. 7.0%, Ki-67: 15.3% vs. 4.0%, γH2AX: 76.6% vs. ±90%, Caspase cleaved cytokeratin 18: ±3% vs. ±12% Addition of cisplatin: 1.9-fold increase in apoptotic index	-	-

5-FU=5-fluoroucil, RT=radiotherapy, FACS=fluorescence-activated cell sorting, ALD=aldehyde dehydrogenase, CSC=cancer stem cell, MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, ALDH1A1=aldehyde dehydrogenase 1 family, member A1, IC50=half maximal inhibitory concentration, MMC=mitomycin C, ^{3}H-TdR=[^{3}H] radiolabeled thymidine, HDRA=Histoculture Drug Response Assay, PPV=positive predictive value, NPV=negative predictive value, pt-DNA=platinum-DNA, CSS=cause-specific survival, THP: 4-0-tetrahydropyranyl adriamycin, ADM=adriamycin, BLM=bleomycin, TPR=true positive ratio, TNR=true negative ratio, I.I=Inhibition Index, MTX=methotrexate, IHC=immunohistochemistry, SUV=standardized uptake value, OS=overall survival, LDH=lactate dehydrogenase, WST-1=4-[3-(4-iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate, AI=apoptotic index, Gy=Gray, TUNEL=terminal deoxynucleotidyl transferase dUTP nick end labeling, CRT=chemoradiotherapy, #=number.

Table 9.4 Overview of selected studies using immunotherapy or targeted therapy on various cultures from HNSCC tissue.

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment
Kross, 2007[70]	Multicellular spheroids	18	14	>90%	LLME	ELISA, LIVE/DEAD kit, BrdU labeling	Nearly 100% of the spheroid surface consisted of live cells, indicating viability after 14 days of culture in vitro.	Mean IL-6 production in 168 h, control vs. treated: ±17.500 vs. ±5000 pg/mL Mean MCP-1 production in 168 h, control vs. treated: ± 7000 vs. ± 1000 pg/mL
Kloss, 2015[71]	Multicellular spheroids	5	11–14	100%	Cetuximab	Cytometric bead array, fluorescent microscopy, FACS	-	When cetuximab was absent, the NK cells showed clearly impaired and disordered "effector-to-target" interactions and decreased both cancer cell cluster infiltrations and cancer cell killing.
Sun, 2014[72]	CSC-enriched spheroids	3	6	-	c-Met inhibitor PF-2341066	Sphere-forming ability	Immunofluorescent staining showed that the spheres have high expression levels of several known CSC markers.	Sphere formation was inhibited in a dose-dependent manner. CSC cells were more sensitive to PF-2341066 than to docetaxel. In contrast, differentiated cells show the opposite effect
Driehuis, 2019[31]	Organoids	34	42	60%	Nutlin-3 Cetuximab, Alpelisib, Vemurafenib, Everolimus, AZD4547 Niraparib	CellTiter-Glo 3-D Assay	Tumor-specific histopathologic changes were retained in culture. Organoids contain only transformed tumor cells.	IC50 nutlin-3: 0.5–22.6 µM AUC cetuximab: 93.94–180.7 IC50 alpelisib: 0.12–4.12 µM IC50 everolimus: 0.00–19.83 µM IC50 AZD4547: 0.67–28.38 µM IC50 niraparib: 4.24–25.66 µM
Dean, 2010[73]	Histocultures (800–1000 µm)	22	3	86.4%	Anti-EMMPRIN mAb, Cetuximab	ATP viability assay, TUNEL	Cultures had excellent viability over 72 h. Less than 5% of any specimen showed necrosis.	Average ATP level anti-EMMPRIN vs. cetuximab: 57% vs. 45% (control: 100%) ($p = 0.13$) Apoptosis was increased in anti-EMMPRIN-treated cultures (77%) vs. controls (30%).
Gerlach, 2013[34]	Histocultures (350 µm)	12	3–6	-	Cetuximab	LDH release, IHC, TUNEL	Slice cultures maintained morphological features for up to 6 days as compared to the original diagnostic histopathology. No change of γH2AX positivity was visible at any of the tested time points.	# nuclei control vs. cetuximab: ±400 vs. ±25 Percentage of caspase-3-positive cells control vs. cetuximab: ±2% vs. ±5%
Freudlsperger, 2014[35]	Histocultures (300–350 µm)	15	6	-	LY294002	IHC	Histological staining confirmed preservation of tissue architecture. The cultures showed almost 100% Ki-67 staining and few apoptotic cells.	Expression after treatment with LY294002 vs. RT vs. LY294002 + RT (control 100%): p-AKT: ±65% vs. ±135% vs. ±55% p-H2AX: ±80% vs. ±900% vs. ±1700% Ki-67: ±80% vs. ±70% vs. ±35%

Table 9.4 (continued)

Authors, Year	Culture Technique	Patients (N)	Culture Duration (Days)	Culture Success (%)	Ex Vivo Treatment	Response Read-Out Method	Preservation of Tissue Parameters in Culture	Main Results of Treatment
Peria, 2015[36]	Histocultures (300 µm)	5	3	80%	Cetuximab, Sorafenib	IHC	After 72 h, an increase in necrosis was observed in cultured tumor slices. After 48 h, proliferation decreased by 30–70%.	Average % Ki-67-positive cells, control vs. cetuximab vs. sorafenib: ±25% vs. ±15% vs. ±21%
Rauth, 2016[74]	Histocultures (2–3 mm^3)	5	3	100%	Lupeol	IHC	Key components of tumor microenvironment were found to be intact up to 3 days.	Tumor cell content control vs. Lupeol: ±70% vs. ±45% (p <0.05) Ki-67-positive cells control vs. Lupeol: ±15% vs. ±2% (p < 0.01)
Affolter, 2016[75]	Histocultures (800–1000 µm)	9	6	100%	MEK inhibitor PD-0325901	IHC	The number of Ki-67-positive tumor cells was 5% to 7.5% in non-treated cultures. In 1 culture, 75% of all cells were positive for Ki-67 in the control. γH2AX expression levels varied widely between 10% and 95%.	Expression after treatment with 0 µM PD-0325901 + 5 Gy vs. 20 µM PD-0325901 + 5 Gy: pERK: 27.8% vs. 4.4% Ki-67: 8.1% vs. 1.8% γH2AX: 43.1% vs. 43.1%
Donnadieu, 2016[76]	Histocultures (300µm)	18	2	78%	Rapamycin Sorafenib Cetuximab Erlotinib Masatinib Ponatinib Afatinib Tivantinib	IHC	-	Average % of cell inhibition (control 100%): Rapamycin: 77.1% Sorafenib: 65.7% Cetuximab: 73.4% Erlotinib: 75.9% Masatinib: 70.5% Ponatinib: 74.2% Afatinib: 60.9% Tivantinib: 80.9%
Al-Samadi, 2019[42]	Microdevice	5	3	-	IDO 1 inhibitor, PD-L1 antibody	Fluorescent microscopy-based cell counting	-	AUC # of infiltrated immune cells Control vs. IDO 1 vs. PDL-L1: Patient 4: ±550 vs. ±850 vs. ±400 Patient 5: ±0 vs. ±250 vs. ±0 AUC cancer cell proliferation rate: Patient 4: ±1.0 vs. ±0.85 vs. ±0.4 Patient 5: ±1.0 vs. ±0.7 vs. ±0.8

LLME=L-leucine-methylester, ELISA=enzyme-linked immunosorbent assay, BrdU=bromodeoxyuridine, F-spheroids=fragment spheroids, IL-6=interleukin-6, MCP-1=monocyte chemoattractant protein-1, FACS=fluorescence-activated Cell Sorting, NK cells=Natural Killer cells, CSC=cancer stem cell, AUC=area under the curve, EMMPRIN=extracellular matrix metalloproteinase inducer, mAb=monoclonal antibody, ATP=adenosine triphosphate, TUNEL=terminal deoxynucleotidyl transferase dUTP nick end labeling, LDH=lactate dehydrogenase, IHC=immunohistochemistry, RT=radiotherapy, and Gy=Gray; #=number.

IT/TT

Three studies of Heimdal and Olsnes describe multicellular spheroids in co-culture with monocytes or monocyte-derived macrophages.[20,77,78] To elucidate the mechanisms of monocyte cytokine secretion, fragment spheroids (F-spheroids) from malignant and benign mucosal tissue were cultured in the presence of monoclonal antibodies against CD14, CD29, and MCP-1, molecules involved in monocyte activation and infiltration. Tumor samples from a total of 24 patients were investigated. The monoclonal antibodies affected cytokine secretion, including MCP-1, IL-6, and TNF-a, but the effect on cancer cell viability or survival have not been investigated. However, the same group showed in a separate study that increased levels of IL-6 in these co-cultures are predictive for disease recurrence in HNSCC patients.[79]

F-spheroids have also been used in a subsequent study of Kross et al.[70] The main goal was to analyze tumor-associated macrophage cytokine secretion by treating the spheroids with L-leucine-methylester (LLME), a drug which selectively induces apoptosis in macrophages, but not in tumor cells. LLME treatment only affected the macrophages and their cytokine secretion, without influencing the viability of the tumor cells within the F-spheroids.

Another study using co-cultures was conducted by the same group.[71] Tissue from five patients was used to co-culture multicellular spheroids with Natural Killer (NK) cells. They determined cytotoxic activity of the NK cells after pre-treatment of the spheroids with cetuximab. NK cells showed clearly improved and more organized function when cetuximab was added, which resulted in a higher percentage of killed tumor cells. This observation supports the suitability of this co-culture model to evaluate treatment response.

CSC-enriched spheroids

Two out of five studies using CSC-enriched spheroids reported success percentages of 6% and 80–100%.[24,26] The amount of time required for the cultures varies between 6 and 17 days, with an average of 12 days.

CT/RT

The first sensitivity testing with CSC-enriched spheroids was conducted by Lim *et al.*, investigating culture response to cisplatin, 5-FU, paclitaxel and docetaxel.[24,25] It was observed that undifferentiated spheroids were more chemo-resistant than differentiated spheroids. As an explanation, they showed that undifferentiated spheroids consisted of 1.74% extra chemo-resistant cells, while this percentage was only 0.11% in differentiated spheroids. A second study confirmed this finding by showing that stem cells grown as spheroids or as an adherent monolayer were relatively more chemo-

resistant compared to the same culture models consisting of differentiated cells.[25] The CSC-enriched culture model is interesting to investigate stem cell behavior and characteristics, but the observed differences in drug response in relation to differentiation state might question whether CSC-enriched spheroids are a representative model for the in vivo situation.
A subsequent study investigated radio-sensitivity and migratory potential of CSC-enriched spheroids derived from five patients.[26] They observed no statistically significant difference in surviving fraction and spheroid migration after treatment with radiation doses up to 10 Gy, compared to the untreated control. This is in line with the findings of the previous studies reporting on the chemo-resistance of CSCs.

IT/TT

To overcome therapy resistance, therapies specifically targeting CSCs in HNSCC are also explored with the use of CSC-enriched spheroids. One study investigated therapeutic inhibition of c-Met, which is identified as a self-renewal marker of CSCs in HNSCC patient-derived tumor xenografts.[80] They showed that CSCs were indeed more sensitive to c-Met inhibitor PF-2341066 than to docetaxel, whereas differentiated cells showed the opposite response.[72]

Organoids

The organoid culture technique is relatively new and has not been extensively investigated for HNSCC yet. Reported success percentages vary from 30.2% to 80%. It is reported that organoids can be established in up to 7 days but may be kept in culture for prolonged time if required.[30,31] Drug testing or passaging is recommended after 10-14 days culturing.[30,31,48,81]

CT/RT

One study investigated response to cisplatin, docetaxel and 5-FU and reported IC50 values for several organoid lines. These organoids showed similar histological patterns and expression levels of vimentin and stem cell markers CD44 and ALDH1A1 when compared to their original tumors.[30] IC50 values from organoid drug treatment in vitro were observed to be similar to the drug response in vivo, after injecting these organoids in mice. Interestingly, another study observed that the successful formation of organoids was significantly associated with poor response to pre-surgical neoadjuvant chemotherapy or chemoradiation in their patients. In addition, IC50 values for 5-FU of the organoids were much higher for organoids after passaging (0.4–1.4 µM vs. 23.6-53.6 µM), which is attributed to an increased CD44 expression and autophagy.[48]

One year later, the Clevers group published two studies using HNSCC organoids with an extensive description of methods and organoid characterization.[31,81] When comparing organoids with the original tumor, they observed that specific histopathological changes were retained in culture. However, the organoids only contained the transformed epithelial tumor cells and not the connective tissue, immune or vessel elements. Drug screens were performed on the organoids and IC50 values were reported for cisplatin and carboplatin, showing differential sensitivity of the organoids to these compounds. Area under the curve (AUC) values were calculated for radiotherapy treatment and compared to clinical response of the patients who received (postoperative) radiotherapy. Interestingly, six out of seven patient outcomes matched with the responsiveness of their respective organoids. The organoid of the seventh patient showed to be resistant to radiotherapy in the in vitro assay, whereas the patient showed no signs of recurrence five months after treatment. Longer follow up should reveal whether this patient relapses in the coming months. Even though this is a small population size, this result shows potential for the use of organoid cultures to predict individual radiotherapy response.

IT/TT

On the basis of mutations detected in their organoids or in HNSCC in general, the same study also tested organoid sensitivity to several targeted therapies, including cetuximab, nutlin-3 (p53-MDM2 inhibitor), alpelisib (PIK3CA inhibitor), vemurafenib (BRAF inhibitor), everolimus (mTOR inhibitor), AZD4547 (FGFR inhibitor), and niraparib (PARP inhibitor).[31] No correlation between EGFR expression and cetuximab response was observed. However, organoids insensitive to cetuximab often carried mutations downstream of EGFR. Increased sensitivity to vemurafenib was observed in a BRAF-mutant organoid line, but no correlation was found between responsiveness to alpelisib and PIK3CA mutations. Although mutations in PARP, mTOR and FGFR were not detected in the organoid lines, variable sensitivities to these compounds were observed.

Histocultures

The success percentage of the histoculture model varies from 59% to 100%, with an average of 90% and a median of 98%, as reported by 21 out of 29 articles. Studies describe a culture duration 1 up to 21 days.[32,49,82] In general, the average culture duration of histocultures was 5 days, with a median of 5 days.

CT/RT

The first two groups reporting on HNSCC histocultures determined sensitivity to cisplatin, 5-FU and mitomycin C treatment (Table 9.3). They observed that viable regions

of the cultures were histologically very similar to the original tumor, although regions with necrotic tumor tissue were observed.[32,58,59] The authors presented IC50 values and all three compounds were able to decrease the ^{3}H-TdR incorporation in different histocultures. Hasegawa *et al*. showed that cisplatin and 5-FU were also able to decrease cell viability by an MTT read-out method.[64] In 2013, Gerlach *et al*. cultured tumor sections on a membrane culture insert.[34] They also observed that the cultures were viable and maintained their typical morphological features in vitro for up to 6 days when compared to the original tumor. DNA double strand breaks and cell proliferation was assessed by γH2AX and Ki-67 expression, respectively. Untreated cultures were found to maintain a high proliferative activity and no change in DNA damage was observed over time. Treatment with cisplatin and docetaxel resulted in apoptotic fragmentation, activation of the apoptosis marker caspase-3, and cell loss within the histocultures.[34]

The first comparison between cisplatin response in culture and in vivo was later performed by one of these groups.[32] In this study, they presented predictive data on sensitivity (71%), specificity (78%), positive predictive value (PPV) (83%) and negative predictive value (NPV) (64%) (Table 9.3). More studies followed, investigating clinical correlation with multiple types of chemotherapy and larger patient groups.[60,62,63,65] Generally, these studies reported a good correlation between ex vivo response and clinical response. Whereas the overall sensitivity was relatively high (79-91%), two studies showed a specificity of approximately 50%.[62,63] One of these studies reported that 17 out of 19 patients tested for individual drugs in vitro received a combination of chemotherapies and even in combination with radiotherapy.[62] In a subsequent study, 97% of the patients received the same drug or combinations of drugs that was studied in vitro, making the interpretation of the clinical correlation more reliable.[65]

In addition to clinical drug response, two studies investigated the correlation between in vitro drug response and patient survival. A significantly greater 2 year cause-specific survival was described when ex vivo cultures were sensitive to 5-FU and cisplatin.[61] In line with this, another study showed that a high efficacy of cisplatin in vitro (Inhibition Index >50) was significantly correlated with a better overall survival.[66]

The most recent study on HNSCC histocultures by Engelmann *et al*. described the longest culture duration so far.[49] With the use of a dermal equivalent (DE), they were able to maintain tumor explants of all their non-HPV-driven HNSCCs up to 21 days in vitro. This DE was composed of healthy human-derived fibroblasts and viscose fibers and served as a scaffold for the tumor sample. The authors could distinguish three growth patterns, including an invasive pattern, showing scattered irregular clusters of tumor cells invading the DE, an expansive growth pattern, showing horizontal tumor cell spreading on top of the DE, and a silent growth pattern, without invasion or horizontal spreading. Treatment of the cultures with radiotherapy showed variable responses

characterized by expression levels of apoptosis (caspase-3-positive cells). Two out of five irradiated samples showed an increase in caspase-3 expression, with both of these samples being HPV driven. Interestingly, one patient developed local relapse 17 months after surgery and radiotherapy, with the corresponding ex vivo culture showing an invasive growth pattern. Unfortunately, sensitivity to ex vivo radiotherapy was not examined on this tumor sample. Importantly, this study described that culturing HPV-driven tumor samples appeared to be more challenging compared to non-HPV-driven tumors. Although they were able to maintain HPV-driven samples for up to 21 days, they observed that half of these cultures showed either decreased levels of p16 or decreased amount of cancer cells on day 14.

IT/TT

Dean *et al*. were the first to report the use of HNSCC histocultures for IT/TT sensitivity testing in 2010.[73] They performed sensitivity testing for cetuximab and a monoclonal antibody against extracellular matrix metalloproteinase inducer (EMMPRIN), a cell surface molecule known to promote tumor growth and angiogenesis in HNSCC. It was observed that tumor sections were viable for up to 72 h and that less than 5% of the specimens showed necrosis. Anti-EMMPRIN therapy resulted in a reduced cell proliferation and an increase in caspase-mediated apoptosis. In addition, a larger percentage of ex vivo cultures was sensitive to the anti-EMMPRIM antibody compared to cetuximab (58% vs. 33%).

Sensitivity testing to cetuximab was investigated by four studies.[34,36,73,76] Three of these studies used smaller tumor slices than described previously (300–350 µm in thickness). Concerning ex vivo tissue viability, contradictory results were presented by these groups. As mentioned earlier, Gerlach and colleagues reported a good tissue viability with a high proliferative activity for up to 6 days, whereas another study observed a 30–70% decrease in cell proliferation after 48 h and after 72 h necrosis has increased significantly without treatment. This resulted in an average of 25% proliferating (Ki-67-positive) cells in the control samples.[36] In general, it was observed that cetuximab decreased cell viability (ATP levels), the number of nuclei, and number of Ki-67-positive cells, while the number of apoptotic (caspase-3-positive) cells was increased.

In addition to cetuximab, other targeted therapies that are not used in clinical practice have been tested on HNSCC histocultures. It was presented that treatment with the PI3K inhibitor LY294002 sensitizes ex vivo cultures to radiotherapy, resulting in increased DNA damage and decreased cell proliferation.[35] No reduction in cell proliferation was observed after treatment of histocultures with the RAF kinase inhibitor sorafenib.[36] Lupeol, a naturally occurring phytochemical found in fruits, vegetables and plants was also tested for its effects on cell viability and proliferation. Lupeol treatment showed

profound decrease in proliferation (Ki-67 expression) compared to control tissues.[74] Ex vivo treatment with the MEK inhibitor PD-0325901, either in combination with radiotherapy or as monotherapy, only showed modest effects on cell proliferation. This might be attributable to the very low proliferation fraction in control tissues in this study (5% to 7.5%). MEK inhibition prior to irradiation decreased p-ERK levels and increased γH2AX levels predominantly in one patient sample with low basal γH2AX expression.[75]
Donnadieu *et al*. cultured tumor slices of HNSCC and exposed them to a panel of targeted therapies.[76] These therapies were selected based on their inhibitory effect on oncogenic kinases and reached phase II/III in clinical trials for the treatment of various solid tumors, including EGFR, B-RAF, KIT, HGFR, FRFR, and mTOR. They observed that effect of treatment varied depending on drug and patient. The multi-kinase inhibitor sorafenib proved to be most effective in inhibiting cell proliferation (5/14 tumors). In total, a more than 50% inhibition of proliferation was observed in 10/14 tumor samples for at least one drug. Although the levels of ERK and p-ERK were determined, no mutational analysis of these oncogenic pathways was described on the ex vivo cultures, which could correlate to drug response.

Microdevices

Whereas microdevices could be designed for the maintenance of various ex vivo culture models, current literature on HNSCC often describes the use for these devices to study histocultures. Success percentages of 67%, 91% and 100% are reported with culture durations varying from 2 to 10 days. One study compared four different culture models with the use of microdevices, including a monolayer, spheroid and histoculture model.[54] This comparison showed the importance of stable culture conditions and revealed that the choice of cell culture format might play a role in the physiology of the cultured cells and outcome of drug sensitivity assays.

CT/RT

Hattersley *et al*. were the first to report on culturing HNSCC tissue with the use of a microdevice and tested sensitivity to cisplatin and 5-FU.[41] The nuclei of the tissue seemed intact after 72 h, and the percentage of viable cells after 7 days was 72% in the control samples. They specified that there was no evidence of central necrosis, which could be attributed to the microfluidic diffusion. A decrease in cell viability (decrease in WST-1 metabolism, increase in LDH release) and induction of apoptosis (increased cytochrome-c release) were observed after treatment with both compounds.
The efficacy of radiotherapy on HNSCC histocultures is also investigated with the use of microdevices.[67,68] The first study observed a significant increase in cell death, measured by LDH release, 2 h after irradiation of the tissues with 40 Gy.[67] Whereas there was no

difference in apoptotic activity (<2%) between control and uncultured tumor samples, a dose-dependent increase in apoptosis was observed in the radiotherapy treated tissues. In line with this, a second study detected increased apoptosis and higher levels of DNA fragmentation after irradiation. Expression of γH2AX was raised after treatment, but not significantly. The percentage of proliferating cells decreased in a dose-dependent way following irradiation. In the same study, the correlation of ex vivo radiosensitivity and clinical response is also investigated. Although clinical information was only available for two patients, matched responses were observed for both patients and their representative ex vivo cultures. Important to mention is that this study used four markers to predict response to radiotherapy in vitro (LDH release, γH2AX expression, CK18-LI, DNA fragmentation, and Ki-67 expression), and for each patient, only two of these markers were matching clinical response. In addition, one of the patients received chemoradiotherapy, whereas only radiosensitivity was determined ex vivo.[68]

A recent study determined radiosensitivity in combination with cisplatin.[69] They observed that γH2AX expression and the number of apoptotic cells were similar in untreated control and pre-culture samples, whereas the cell proliferation (Ki-67) had decreased in control samples when compared to the pre-culture samples. Irradiation reduced proliferation (BrdU), increased DNA damage (γH2AX), and caspase-dependent apoptosis (caspase-cleaved cytokeratin-18). Caspase-dependent apoptosis was further increased by concurrent cisplatin treatment.

IT/TT

Microdevices are also used as a co-culture system with immune cells to examine immune cell migration and cancer cell proliferation in response to an PDL-1 antibody and IDO 1 inhibitor.[42] This study showed that IDO 1 inhibitor, but not PD-L1 inhibitor, induced immune cell migration towards cancer cells. Drug efficacy on cell proliferation was variable between the two tumor samples from HNSCC patients. Since immune cell migration did not parallel the effect on cancer cell proliferation, it is considered that immune cell migration is not sufficient to evaluate therapy response to immunotherapeutic drugs in this setting.

Discussion

With this review, we aim to provide a comprehensive overview of the current literature on ex vivo 3D culture techniques for HNSCC and evaluate their suitability as a preclinical prediction assay for individualized therapy selection. With the increasing knowledge on driver mutations and deregulated cellular pathways in HNSCC, the development of new

(targeted) treatments, and the varying response rates for both standard-of-care and new therapies, a reliable prediction assay for therapy response is more important than ever.

When culturing primary tissue or cells in general, multiple aspects need to be considered in relation to culture success percentage. It is essential to minimize the time between surgery and start of the culture, since cutting of the blood supply (ischemia) could lead to fast deterioration of the tissue.[83] Furthermore, primary cells have a limited lifespan and are more sensitive to environmental changes and stress compared to immortalized cell lines. In addition, primary cultures are prone to microbial contaminations with bacteria and/or fungi, especially when the tissue is derived from locations with an extensive microbiome, such as the intestines or oral cavity.[84,85] Contamination with fibroblasts could also be a practical challenge, since fibroblasts are able to overgrow the culture because of their high proliferation rate.[86,87] For primary cultures of HNSCC specifically, it remains a challenge to successfully culture and maintain HPV-positive cells and tissues in vitro.[49,88,89] The exact explanation is still unknown, but it is thought that tumor cells must have acquired traits or mutations compatible with survival and immortality to be able to survive in the unnatural in vitro environment. This is supported by the fact that almost all currently used HPV-positive HNSCC cell lines are from smoking patients with aggressive tumors that fail to respond to initial therapy.[88,90] Furthermore, the stromal microenvironment is thought to be involved, if not essential, in HPV-positive epithelial cell growth and disease initiation and maintenance by reciprocal epithelial-stromal interactions.[91,92] Thus, HPV-positive tumor cells might require the presence of (specific factors within) the microenvironment in order to survive in vitro. Recent data have shown that HPV-positive tumors are a heterogeneous group and can be subclassified based on genomic profiles (e.g., characterized by a signature of mesenchymal and immunological response genes (HPV-IMU), or keratinocyte differentiation and oxidative stress genes (HPV-KRT), with the latter subgroup showing more frequently integrated HPV and enrichment of PI3KCA mutations), EGFR expression, and HPV integration status, amongst others.[93-95] In addition to patient prognosis, these factors might also influence in vitro viability of HPV-positive tissues. In the investigated literature, specific information on virus positivity of the tumor in relation to ex vivo culture success rate is limited.

One of the essential requirements for a reliable tumor model is the resemblance to the original tumor composition as closely as possible, since the tumor-microenvironment, including multiple cell types and tumor-stroma interactions, has shown to influence tumor behavior and therapeutic response.[96,97] In addition, culture success rate and

culture duration are important aspects for a tumor model to serve as a preclinical prediction assay. In this review, we show that the best culture success rates have been achieved with the histoculture technique. Furthermore, as this culture method does not require enzymatic dissociation, natural tumor heterogeneity, cell–cell interactions and cell-stroma interactions are left intact, resulting in the best simulation of the in vivo situation as possible. An additional advantage might be the relatively short-term culture duration of this tumor model which reduces the chance on phenotypic and genetic alterations, as observed in more long-term cultures, allowing for fast decision making in a personalized therapy approach. In contrast, the often relatively quick occurrence of tissue deterioration during culturing might influence the outcome of drug sensitivity assays.[36] Improving tissue viability over time, for example by the use of a dermal equivalent (consisting of viscose fibers and human-derived fibroblasts) as tissue support, could increase reliability of the histoculture model and allow for prolonged ex vivo drug exposure.[49] In addition, microdevices might offer a chance to increase tissue viability by providing a controlled culture environment and continuous perfusion and nutrient supply to the tumor tissue. However, there is no convincing evidence yet for the role of microdevices in prolonging HNSCC tissue viability compared to conventional culture methods.

Longer culture durations are reported for HNSCC spheroid and organoid models evaluated in this review. Organoids specifically can be expanded for a long period of time and cryopreserved, which allows for a wide range of research applications, such as genetic modification and a prolonged exposure to anti-cancer drugs.[98] In addition, less tumor material is required for organoid generation, compared to histocultures. However, organoids only comprise of (transformed) epithelial cells, without native micro-environment with stromal compartment, immune cells, nervous system, and vessel elements.[31,99] The possibility to co-culture these models might offer an opportunity to overcome this limitation and study the interaction between different cell types. The same limitation is observed for spheroid models, especially when cultures are enriched for CSCs. Whereas this model could be interesting to investigate stem cell characteristics and behavior in relation to drug resistance, the resemblance to the original tumor might be questioned. In addition, identification and isolation of stem cells from tissues remains a challenge and is often based on stem cell markers, such as CD44 and ALDH. However, none of these stem cell markers has proven to identify CSCs with adequate sensitivity and specificity. Besides this, there are other unresolved aspects, such as the impure and variable stem cell population in human tumors and the stability of CSC immunophenotype over time.[100,101] Whereas there is a selection for cell type in organoids and CSC-enriched spheroids, the organotypic multicellular spheroids (or F-spheroids) are established by only mechanically modification of tissues, similar to

histocultures. In contrast, these F-spheroids were cultured to form rounded spheres before use in sensitivity assays, which took typically 10–14 days.[20,70,77,78] During this period of spheroid generation, loss of epithelial cells from 28% to 12.9% was observed by one of these groups in a separate study.[102]

In addition to aforementioned technical considerations, a preclinical prediction assay should be able to accurately predict patient therapy response. So far, clinical correlations were mainly reported by studies investigating sensitivity to chemotherapy with the use of ex vivo histocultures. Overall, this technique shows good predictive values (accuracy of 74–79%). However, two out of four studies describe a specificity of approximately 50%.[62,63] This means that half of the cultures were sensitive to chemotherapy ex vivo, while the corresponding patient showed no clinical response. This might be explained by mechanisms of resistance in vivo in addition to those at cellular level, for example the variation in pharmacokinetics between different patients. If the tumor cells are highly resistant ex vivo, there is a small chance that the drug will be effective in vivo. Therefore, it is argued that these (chemo)sensitivity assays might be a better predictor for therapy resistance than sensitivity.[103] Whereas increased tumor response rates do not necessarily increase patient survival, evidence is needed from clinical trials investigating patient survival in correlation to ex vivo drug sensitivity. Two studies investigated this correlation and reported a better cause-specific survival and overall survival when histocultures were sensitive to chemotherapy.[61,66] However, only chemosensitivity was tested ex vivo, while patients in both studies often received a combination of treatments with radiotherapy and/or surgery, which might cause a bias in survival data.

Evidence for the predictive value of organoid and spheroid models of HNSCC is still sparse. One recent study showed a correlation between organoid radiosensitivity and clinical responses in six out of seven patients.[31] Although the number of patients is small and these tumors comprise a heterogeneous group, all patients were treated with (postoperative) radiotherapy only. This allows for a reliable comparison between ex vivo and patient response. An ongoing study of the same group aims to include approximately 80 patients to follow up on these initial findings and elucidate whether organoid responses hold predictive potential for patient responses.

Although immunotherapy has become a new promising treatment modality for HNSCC with varying response rates, ex vivo sensitivity to these therapies has not been correlated to clinical response in the reviewed literature. Multiple studies do show the possibility to maintain and include immune cells in culture, which is essential to assess immunotherapy sensitivity.[42,70,71] With the exception of cetuximab, therapies targeting specific mutations are not routinely used to treat HNSCC patients yet. This makes it

difficult to correlate ex vivo findings to the clinical situation. Nevertheless, it is of interest to investigate mutation status of the tumor in correlation to ex vivo sensitivity to targeted therapies.[31,104] In this context, Driehuis *et al*. observed increased sensitivity to a BRAF inhibitor in a BRAF-mutant organoid line derived from a BRAF-mutant HNSCC. In other cases, no correlation was observed, for example between organoid EGFR expression and cetuximab response and the presence of PIK3CA mutations and the responsiveness to PI3K inhibitor alpelisib.[31]

As ex vivo cell culture models have matured in recent years, they have not become part of clinical routine yet. For this purpose, efforts should be made to improve technical aspects of all culture models in order to more closely resemble the original tumor (-environment), increase ex vivo cell viability and culture success rates, also for HPV-positive tumors. The presence of immune components in culture is not only essential for evaluating immunotherapy sensitivity, but may also influence sensitivity to other therapies, including chemotherapy, radiotherapy and targeted therapies.[105-107] In addition, larger studies should focus on obtaining more evidence on the predictive potential of ex vivo models with both tumor response and patient survival, in which ex vivo and in vivo treatment should be similar to allow for a reliable comparison and prediction. In addition, the application of testing targeted therapies would be most interesting for those tumor subtypes that require additional treatment or are characterized by an unfavorable prognosis, for example caused by radioresistance. Lastly, the use of unambiguous terminology should be a prerequisite for all studies reporting on 3D culture techniques. This will ensure that evaluating and comparing future research as well as working towards the best preclinical prediction model will be improved.

Conclusions

There is a strong need for preclinical 3D models of HNSCC, which allow prediction of therapeutic response in a personalized setting and furthermore enable novel drug testing before introduction into clinical practice. In this review, we observed that a wide range of ex vivo culture techniques have been introduced for HNSCC, all with their own advantages, limitations, and applications. So far, most information is available on HNSCC histocultures and their use to obtain an indication for response to chemotherapy. Future research should elucidate whether histocultures and/or other ex vivo tumor models can mature further to useful clinical tools.

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Table S9.1 Overview of included studies describing ex-vivo culture models of HNSCC for chemotherapy and radiotherapy sensitivity testing.

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Leong, 2014[51]	Multicellular Spheroid	3	4-9	-	Cisplatin, 5-FU, Etoposide, RT	FACS	-	Spheroids were more resistant to all treatments than monolayers. Cells with a high ALD expression were resistant to cytotoxic agents.	-	-
Hagemann, 2017[22]	Multicellular Spheroid	-	15	-	Cisplatin, 5-FU, RT	Spheroid area and diameter, ELISA	In preliminary data, primary cells did not form reproducible spheroids in hanging-drop but did in ultra-low attachment plates.	Cisplatin, 5-FU, and RT significantly decrease spheroid growth Combination of RT and cisplatin reduced number of viable cells but not spheroid size.	-	-
Lim, 2011[24]	CSC-enriched spheroids	47	14	6%	Cisplatin, 5-FU, Paclitaxel, Docetaxel	MTT	-	Undifferentiated spheroid cells were significantly more resistant to all chemotherapeutic agents than differentiated spheroid cells.	-	-
Lim, 2012[25]	CSC-enriched spheroids	31	15-17	-	Cisplatin, 5-FU, Paclitaxel, Docetaxel	MTT	-	Cell survival: Control 100% Cisplatin: 70% 5-FU: 60% Paclitaxel: 55% Docetaxel: 60%	-	
Oh, 2013[104]	CSC-enriched spheroids	-	>14	-	Cisplatin	MTT	-	CD44+ and CD44− cells were resistant to cisplatin, as compared to differentiated spheroids.	-	-
Kaseb, 2016[26]	CSC-enriched spheroids 5	5	7-11	80 – 100%	RT	Colony forming assay, spheroid migration assay	-	SF 2.5 Gy: 80% SF 5 Gy: 75% Spheroid migration 0 Gy: 2000%; 2.5 Gy: 1400%; 5 Gy: 1100%	-	-
Kijima, 2018[48]	Organoids	5	14	80%	5-FU, Chloroquine	WST-1 metabolism, IHC, FACS	Organoids recapitulated histopathology of the original tumor. 5-FU resistance has increased in secondary (p1) organoids compared to primary (p0) organoids	IC50 5-FU p0: 1.360 – 0.925 – 0.389 µM IC50 5-FU p1: 23.60 – 53.62 µM	-	-

Table S9.1 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Tanaka, 2018[30]	Organoids	43	8 - 30	30.2%	Cisplatin, Docetaxel	Relative organoid area day 1 *vs.* day 8	Histological patterns, vimentin expression and CD44/ALDH1A1 ratios were similar between organoids and the original tumor.	Cisplatin IC50: 0.92 – 1.02 µM Docetaxel IC50: 1.46 – 3.75 nM	-	-
Driehuis, 2019[31]	Organoids	34	42	60%	Cisplatin, Carboplatin, RT	CellTiter-Glo 3-D Assay	Tumor-specific histopathologic changes were retained in culture. Organoids contain only transformed tumor cells.	IC50 cisplatin: 0.5 – 12.8 µM IC50 carboplatin: 3.0 – 81.9 µM AUC RT: 238 - 698	RT	6/7 matched response: 3 positive outcomes with sensitive organoid, 3 no response with non-sensitive organoid.
Robbins, 1991[77]	Histocultures (1-2 mm^3)	15	3-11	67%	Cisplatin, 5-FU	^{3}H-TdR	-	Cisplatin sensitive: 5/10 5-FU sensitive: 4/9 Cisplatin + 5-FU sensitive: 7/8	-	-
Au, 1993[52]	Histocultures (HDRA)	83	9	59%	Cisplatin, 5-FU, MMC	^{3}H-TdR	Most histocultures contained areas of viable and necrotic tissue. Histology of viable regions of the histocultures was similar to that of the fresh tumor.	Primary tumors mean IC50: 5-FU: 0.68 ± 0.74 µg/ml Cisplatin: 3.77 ± 2.42 µg/ml MMC: 0.25 ± 0.13 µg/ml 9/47 tumors not sensitive	-	-
Robbins, 1994[32]	Histocultures (HDRA)	26	3-11	88%	Cisplatin	^{3}H-TdR	-	84% reduction in the number of cells incorporating ^{3}H-TdR in in drug-treated samples compared to control samples is used as the cutt-off for sensitivity *in-vitro*.	Cisplatin	Sensitivity: 71% Specificity: 78% PPV: 83% NPV: 64%

Table S9.1 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Robbins, 1996[53]	Histocultures	43	6-9	91%	Cisplatin	^{3}H-TdR	-	Sensitivity overall: 1.5µg/ml: 22% 15 µg/ml: 62% 37.5 µg/ml: 83% Factor growth inhibition: Untreated lesions: x 2.44 Recurrent tumors: x 5.56	-	-
Kuh, 1999[105]	Histocultures (1 mm^{3})	3	2-4	-	Paclitaxel	[3H]paclitaxel uptake and efflux	-	T ½, uptake: 12 nM: 24.2 hours 120 nM: 19.8 hours 1200 nM: 10.4 hours 12,000nM: 5.81 hours T ½, efflux: 120 nM: 7.45 hours 1200 nM: 3.33 hours	-	-
Welters, 1999[54]	Histocultures (3 mm^{3})	8	1	-	Cisplatin	32-P labeling	-	Because most of the biopsies were too small to perform analyses at several time points, no adduct levels over time could be measured.	Cisplatin	DNA adduct levels partial responder *vs* non- responder: Pt-GG: 27.4 *vs*. 5.1 Pt-AG: 13.2 *vs.* 2.4
Singh, 2002[55]	Histocultures (HDRA)	41	2	98%	Cisplatin, 5-FU	MTT	-	Number of resistant tumors: 13/41 resistant to 5-FU 13/41 resistant to cisplatin 11/41 resistant to both	Cisplatin, 5-FU, RT	2-year CSS sensitive *vs* not-sensitive: 5-FU: 85% *vs*. 64% Cisplatin: 86% *vs.* 63% 5-FU + cisplatin: 85% *vs.* 63%

Table S9.1 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Ariyoshi, 2003[56]	Histocultures (HDRA)	19	7	100%	Cisplatin, Docetaxel, 5-FU, BLM THP, TXT ADM	MTT	-	Sensitivity rate per drug: Cisplatin: 78.9% 5-FU: 38.4% BLM: 21.4% THP: 7.7% TXT: 100% ADM: 0%	Cisplatin, 5-FU, THP, BLM	Accuracy: 78.9% Sensitivity: 86.7% Specificity: 50% TPR: 86.7% TNR: 50%
Gan, 2006[106]	Histocultures (1 mm^3)	22	3-4	100%	Paclitaxel	BrdU labeling, 3-TH, TUNEL, DNA fragmentation analysis, IHC	-	Max. antiproliferation effect: 40%, Max. apoptotic index: 12% Max. overall effect (max. inhibition of DNA labeling index in non-apoptotic cells): 60%	-	
Hasegawa, 2006[57]	Histocultures (HDRA)	49	7	100%	Cisplatin, 5-FU	MTT	-	Cisplatin efficacy rate: 36.7-71.4% 5-FU 120µg/ml *vs.* 300µg/ml efficacy rate: 23.1-57.7% *vs.* 70.8-75.0%	Cisplatin, 5-FU	Prediction rate: 77.8% Sensitivity: 90.9% Specificity: 57.1% TPR: 76.9% TNR: 80.0%
Hasegawa, 2008[58]	Histocultures	44	7	82%	Cisplatin, 5-FU	MTT	-	Mean I.I. 5-FU: 36.76% Mean I.I. cisplatin: 35.65% 5-FU sensitivity: 21/44 (58.3%) Cisplatin sensitivity: 21/44 (58.3%)	-	-
Pathak, 2008[59]	Histocultures (HDRA)	57	8	91%	Cisplatin, 5-FU, MTX	MTT	-	cisplatin sensitivity: 52% 5-FU sensitivity: 46% MTX sensitivity: 52% Sensitive to one drug: 88%	Cisplatin, 5-FU, MTX, Paclitaxel, Ifosfamide	Accuracy: 74% Sensitivity: 79% Specificity: 71% PPV: 69% NPV: 80%
Gerlach, 2013[34]	Histocultures (350 µm)	12	3-6	-	Cisplatin, docetaxel	IHC	Cultures maintained morphological features and γH2AX expression for up to 6 days compared to original histopathology.	Control *vs.* cisplatin *vs* docetaxel: # nuclei: ±400 *vs.* ±125 *vs.* ±150 % caspase-3 positive cells: ±2% *vs.* ±6% *vs.* ±22%	-	-

Table S9.1 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Yu, 2014[107]	Histocultures	2	-	-	5-ALA-PDT	FACS	-	ALDH1 activity: control: 100% ALA-PDT treated cell line *vs* tissue slice: 60% *vs*. 45%	-	-
Suzuki, 2015[60]	Histocultures (HDRA)	28	7	100%	Cisplatin	MTT	-	SUV_{max}: 14.04 ± 7.52 I.I.: 50.98 ± 26.6 SUV_{max} was significantly correlated with the I.I. cisplatin (p<0.04, R^2=0.17)	Cisplatin, 5-FU, RT	SUV_{max} ≥10.5 and I.I. cisplatin <50 were significantly correlated with shorter OS
Engelmann, 2020[61]	Histocultures	13	7-21	100%	RT	IHC	Comparable histological and morphological characteristics were observed between primary non-HPV driven tumors and histocultures after 14 days. Cultures display heterogeneous growth patterns on dermal equivalent	Irradiation of tissues resulted in a slight increase or decrease in Ki-67 expression compared to control: Overall: + 0.22% Non-HPV driven: -5.28% HPV-driven: +3.89% 2/5 tumors showed increase in apoptotic cells after fractionated irradiation	RT	One patient developed local relapse, with the corresponding histoculture showing an invasive growth pattern.
Hattersley, 2012[41]	Microdevice	23	8	91%	Cisplatin, 5-FU	LDH and cytochrome c release, WST-1 metabolism	The nuclei of the tissue 72h after culture appear intact and loss of cell cohesion is minimal. There was no necrosis in the center of the biopsy.	% viable cells after treatment: Control: 72% ± 15.6 5-FU: 45% ± 22.3 Cisplatin: 44% ± 20.2 5-FU + Cisplatin: 30% ± 23.7 All treatments showed a higher release of cytochrome-c than the control samples. ($p < 0.01$)	-	-
Sylvester, 2013[108]	Microdevice	3	10	-	Cisplatin, 5-FU, Docetaxel	LDH release assay, WST-1 metabolism	The combination of LDH release and WST-1 metabolism demonstrated tissue viability within the device.	Cisplatin alone and combination treatments were significantly different from the untreated controls and 5-FU samples.	-	-

Table S9.1 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment	In-patient treatment	Correlation ex-vivo *vs* in-patient
Carr, 2014[62]	Microdevice	35	2-3	-	RT	LDH and cytochrome c release, IHC	There was no significant difference between the apoptotic index (AI) of the uncultured and cultured control tissue (p = 0.29).	AI 0 Gy: ±1% AI 5 Gy: ±7% AI 10 Gy: ±15% AI 20 Gy: ±20% AI 40 Gy: ±45% (p=0.006)	-	-
Hsieh, 2015[78]	Microdevice	-	2	-	Cisplatin	Cell Counting Kit 8, LIVE/DEAD kit,	The metabolic activitity of cells in the 3D culture were higher than in the spheroid culture. The proliferation of the primary cells in the 3D culture was about 11–101% higher than that in the 2D and spheroid cultures.	Spheroid cultures might overestimate chemoresistance to cisplatin, the 2D and 3D culture models might overestimate the chemosensitivity to cisplatin The chemosensitivity of primary cells in the tumor tissue and the 3D culture models showed no statistical difference, indicating that chemosensitivity of the 3D culture was closer to that of the native tumor.	-	-
Cheah, 2017[63]	Microdevice	5	2	100%	RT, Cisplatin	LDH release, IHC, TUNEL	-	γH2AX: 1/5 sign. response CK18-LI: 2/5 sign. response TUNEL: 3/4 sign. response Ki-67: 0/5 sign. response	RT, CRT	Matched responses for 2/2 patients (for 2/4 markers)
Kennedy, 2019[64]	Microdevice	18	3	67%	RT, Cisplatin	IHC	The average Ki-67 index decreased in the control sample (7.9%±3.5) relative to the pre-culture sample. No difference in γH2AX and apoptosis between pre-culture and control samples.	Control *vs*. RT: BrdU: 13.3% *vs* 7.0%, Ki-67: 15.3% *vs* 4.0%, γH2AX: 76.6% *vs*. ± 90%, Caspase cleaved cytokeratin 18: ± 3% *vs*. ± 12% Addition of cisplatin: 1.9-fold increase in apoptotic index	-	-

5-FU=5-fluoroucil, RT=radiotherapy, FACS=fluorescence-activated cell sorting, ALD=aldehyde dehydrogenase, CSC=cancer stem cell, MTT=3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, SF=surviving fraction, Gy=gray, WST-1=4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene sulfonate, IHC=immunohistochemistry, IC50=half maximal inhibitory concentration, ALDH1A1=aldehyde dehydrogenase 1 family, member A1, AUC=area under the curve, MMC=mitomycin C, 3-TH=[^{3}H]Thymidine, HDRA=histoculture drug response assay, PPV=positive predictive value, NPV=negative predictive value, T ½=time for the tumor concentration to reach 50% of steady-state level, pt-DNA=platinum-DNA, CSS=cause specific survival, BLM=bleomcyin THP: 4-0-tetrahydropyranyl adriamycin, ADM=adriamycin, TPR=true positive ratio, TNR=true negative ratio, BrdU=bromodeoxyuridine, TUNEL=terminal deoxynucleotidyl transferase dUTP nick end labeling, I.I=inhibition index, MTX=methotrexate, 5-ALA-PDT=5-aminolevulinic acid photodynamic therapy, SUV=standardized uptake value, OS=overall survival, LDH=lactate dehydrogenase, AI=apoptotic index, CRT=chemoradiotherapy.

Table S9.2 Overview of included studies describing ex-vivo culture models of HNSCC for immunotherapy and targeted therapy sensitivity testing.

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment
Heimdal, 2001 [20]	Multicellular Spheroids	3	10-14	-	Anti-CD14 mAb	ELISA	-	Addition of anti-CD14 mAb reduces LPS-induced IL-6 secretion. The addition of anti-CD14 mAb to co-cultures of monocytes and spheroids increased the IL-6 response as tested in co-cultures from three donors.
Heimdal, 2001[72]	Multicellular Spheroids	11	10-14	-	anti-MCP-1 mAb	ELISA	-	Addition of anti-MCP-1 mAb decreases co-culture-induced monocyte IL-6 secretion in 4/5 cultures. compared to control cultures.
Olsnes, 2002[73]	Multicellular Spheroids	10	10-14	100%	PFA, D-galactose, D-glucose, D-mannose, D-fructose, anti-CD29 mAb	ELISA	-	Monocyte IL-6 secretion was inhibited by PFA, D-Glucose, D-mannose and anti-CD29 treatment.
Kross, 2007[65]	Multicellular Spheroids	18	14	> 90%	LLME	ELISA, LIVE/DEAD kit, BrdU labeling	Nearly 100% of the spheroid surface consisted of live cells, indicating viability after 14 days of culture in vitro.	Mean IL-6 production in 168 h, control *vs* treated: ± 17.500 *vs*. ± 5000 pg/mL Mean MCP-1 production in 168 h, control *vs* treated: ± 7000 *vs*. ± 1000 pg/mL
Baumeister, 2009[109]	Multicellular Spheroids	30	21	50-100%	Benzopyrene diolepoxide (BPDE), TGF-a	Comet-assay	Overall cell viability was >90%.	Non-treated OTM: 0.9 Control *vs*. tumor BPDE: 7.5 *vs*. 9.4 Control *vs*. tumor BPDE + TGF-a: 7.0 *vs*. 5.7 Control *vs*. tumor hydrogen: 9.7 *vs*. 12.1 Control *vs*. tumor hydrogen + TGF-a: 8.8 *vs*. 11.4 Control *vs* tumor DNA repair capacity: 57.5% *vs*. 45.0%
Leong, 2014[51]	Multicellular Spheroids	3	4-9	-	Gefitinib NVP-AEW541	FACS	-	Tumor spheres were more resistant to all treatment regimens than cells grown in monolayer culture. Although the ALD+ fractions were significantly reduced after treatment with the individual inhibitors, maximal reduction was seen after combined treatment with both drugs

Table S9.2 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment
Kloss, 2015[66]	Multicellular Spheroids	5	11-14	100%	Cetuximab	Cytometric Bead Array, fluorescent microscopy, FACS	-	When cetuximab was absent the NK cells showed clearly impaired and disordered “effector- to target” interactions and decreased both cancer cell cluster infiltrations and cancer cell killing.
Sun, 2014[67]	CSC-enriched spheroids	3	6	-	c-Met inhibitor (PF-2341066)	Sphere forming ability	Immunofluorescent staining showed that the spheres have high expression levels of several known CSC markers	Sphere formation was inhibited in a dose-dependent manner. CSC cells were more sensitive to PF-2341066 than to docetaxel. In contrast, differentiated cells show the opposite effect.
Driehuis, 2019[31]	Organoids	34	42	60%	Nutlin-3 Cetuximab, Alpelisib, Vemurafenib Everolimus, AZD4547 Niraparib	CellTiter-Glo 3-D Assay	Tumor-specific histopathologic changes from the primary material were retained in culture. The organoids contain only epithelial tumor cells, not the immune, connective tissue, or vessel elements.	IC50 Nutlin-3: 0.5 – 22.6 µM AUC cetuximab: 93.94 - 180.7 IC50 alpelisib: 0.12 – 4.12 µM IC50 everolimus: 0.00 – 19.83 µM IC50 AZD4547: 0.67 – 28.38 µM IC50 Niraparib: 4.24 – 25.66 µM
Driehuis, 2019[76]	Organoids	7	7-14	-	PDT (7D12-PS, 7D12-9G8-PS, Cetuximab-PS)	CellTiter-Glo 3-D Assay	EGFR expression levels in organoids were comparable to EGFR levels of primary patient material samples.	AUC of 7D12-PS: 11,490 – 17,657 AUC of 7D12-9G8-PS: 12,167 – 53,548 AUC of Cetuximab-PS: 36,296 – 66,487 EGFR expression levels correlate with response to EGFR-targeted PDT: R^2 = 0.44 (7D12) R^2 = 0.27 (7D12-9G8) R^2 = 0.48 (Cetuximab)
Dean, 2010[68]	Histocultures (800-1000 µm)	22	3	86,4%	Anti-EMMPRIN mAb, Cetuximab	ATP viability assay, TUNEL	Cultures had excellent viability over 72h. Less than 5% of any specimen showed necrosis.	Average ATP level anti-EMMPRIN *vs*. Cetuximab: 57% *vs*. 45% (Control: 100%) (p=0.13) Apoptosis was increased in CNTO treated cultures (77%) *vs*. controls (30%).
Gerlach, 2013[34]	Histocultures (350 µm)	12	3-6	-	Cetuximab	LDH release, IHC, TUNEL	Slice cultures maintained morphological features for up to 6 days as compared to the original diagnostic histopathology. No change of γH2AX positivity was visible at any of the tested time points.	Number of nuclei control *vs*. Cetuximab: ± 400 *vs*. ± 25 Percentage of caspase-3 positive cells control *vs*. Cetuximab: ± 2% *vs*. ± 5%

Table S9.2 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment
Radhakrishnan, 2013[110]	Histocultures	22	3	-	Rapamycin MK-2206	Cell Counting Kit 8, WST assay, IHC	-	Cell viability Rapamycin *vs.* MK-2206 *vs.* combination (control = 100%): 120% *vs.* 100% *vs.* 85% Apoptotic cells Rapamycin *vs.* MK-2206 *vs.* combination (control = 100%): 15% *vs.* 14% *vs.* 19%
Saussez, 2014[111]	Histocultures	5	1	60%	Glutamate, Dopamine, GnRH-II	FACS	The T cells of three patients varied in their basal level of spontaneous and chemotactic migration.	All neurotransmitters increased T-cell migration, CD3zeta expression, and CD3 epsilon expression
Dayekh, 2014[112]	Histocultures (1 mm)	4	2	100%	Monensin	qRT-PCR,	-	3/4 cultures showed >2-fold increase of HMG-CoA reductase mRNA levels after Monensin treatment. 2/4 cultures showed >4-fold increase of ATF3 mRNA levels after Monensin treatment.
Freudlsperger, 2014[35]	Histocultures (300-350 µm)	15	6	-	LY294002	IHC	Histological staining confirmed preservation of tissue architecture. The cultures showed almost 100% Ki-67 staining and few apoptotic cells.	Expression after treatment with LY294002 *vs.* RT *vs.* LY294002 + RT (control 100%): p-AKT:± 65% *vs.* ± 135% *vs.* ± 55% p-H2AX: ± 80% *vs.* ± 900% *vs.* ±1700% Ki-67: ± 80% *vs.* ± 70% *vs.* ±35%
Bourouba, 2015[113]	Histocultures	20	1	-	Anti-TNFa mAb, NOS2 inhibitor	IHC, NO2 measurement (Griess reaction)	-	Average NO2 production anti-TNFa *vs.* 1400W (control = 100%): ± 65% *vs.* ± 55% Ki-67 % proliferation index untreated *vs.* anti-TNFa: ± 45% *vs.* ± 33% (p<0.05)
Peria, 2015[36]	Histocultures (300 µm)	5	3	80%	Cetuximab Sorafenib	IHC	After 72 hours, an increase in tumor necrosis was observed in cultured tumor slices. After 48h, proliferation decreased by 30-70%.	Average % Ki-67 positive cells, control *vs.* Cetuximab *vs.* Sorafenib: ± 25% *vs.* ± 15% *vs.* ± 21%
Rauth, 2016[69]	Histocultures (2-3 mm^3)	5	3	100%	Lupeol	IHC	Key components of tumor microenvironment were found to be intact up to 3 days	Tumor cell content control *vs* Lupeol: ±70% *vs.* ±45% (p<0.05) Ki-67 positive cells control *vs* Lupeol: ± 15% *vs.* ± 2% (p<0.01)
Affolter, 2016[70]	Histocultures (800-1000 µm)	9	6	100%	MEK inhibitor PD-0325901,	IHC	The number of Ki-67 positive tumor cells was 5% to 7.5% in nontreated cultures. In 1 culture, 75% of all cells were positive for Ki-67 in the control. γH2AX expression levels varied widely between 10% and 95%	Expression after treatment with 0 µM PD-0325901 + 5 Gy *vs.* 20 µM PD-0325901+ 5 Gy: pERK: 27.8% *vs.* 4.4% Ki-67: 8.1% *vs.* 1.8% γH2AX: 43.1% *vs.* 43.1%

Table S9.2 (continued)

Authors, year	Culture technique	Patients (N)	Culture duration (days)	Culture success (%)	Ex-vivo treatment	Response read-out method	Preservation of tissue parameters in culture	Main results of treatment
Affolter, 2016[70]	Histocultures (800-1000 µm)	9	6	100%	MEK inhibitor PD-0325901,	IHC	The number of Ki-67 positive tumor cells was 5% to 7.5% in nontreated cultures. In 1 culture, 75% of all cells were positive for Ki-67 in the control. γH2AX expression levels varied widely between 10% and 95%	Expression after treatment with 0 µM PD-0325901 + 5 Gy *vs.* 20 µM PD-0325901+ 5 Gy: pERK: 27.8% *vs.* 4.4% Ki-67: 8.1% *vs.* 1.8% γH2AX: 43.1% *vs.* 43.1%
Donnadieu, 2016[71]	Histocultures (300 µm)	18	2	78%	8 different drugs, see "result treatment column"	IHC	-	Average % of cell inhibition (control = 100%): Rapamycin: 77.1% Sorafenib: 65.7% Cetuximab: 73.4% Erlotinib: 75.9% Masatinib: 70.5% Ponatinib: 74.2% Afatinib: 60.9% Tivantinib: 80.9%
Bhattacharyya, 2017[114]	Histocultures	20	3	-	Lupeol	IHC	-	% proliferation control *vs.* Lupeol: 100% *vs.* ±55% % apoptotic cells control *vs.* Lupeol: ± 1% *vs.* ±11%
Baird, 2018[115]	Histocultures (2mm^3)	5	1	100%	STING ligands	Cytokine secretion analysis, IHC	-	Treatment with STING ligands resulted in increases of IFNα secretion from the explant 4/5 patients showed increased in secretion of CCL3 after treatment with STING ligands.
Carter, 2019[116]	Histocultures (1-2 mm^3)	10	2	100%	BCL-2 family inhibitors (A-1331852 and S63845)	IHC	-	% of cleaved PARP positive tumor cells: Control: 8% A-1331852: 7% S63845: 6% A-131852 + S63845: 36%
Al-Samadi, 2019[42]	Microdevice	5	3	-	IDO 1 inhibitor, PD-L1 antibody	Fluorescent microscopy-based cell counting	-	AUC # of infiltrated immune cells Control *vs.* IDO 1 *vs.* PD-L1: Patient 4: ± 550 *vs.* ± 850 *vs.* ± 400 Patient 5: ± 0 *vs.* ± 250 *vs.* ± 0 AUC cancer cell proliferation rate: Patient 4: ± 1.0 *vs.* ± 0.85 *vs.* ± 0.4 Patient 5: ± 1.0 *vs.* ± 0.7 *vs.* ± 0.8

mAb=monoclonal antibody, ELISA=enzyme-linked immunosorbent assay, LPS=lipopolysacharide , IL-6=interleukin-6, MCP-1=monocyte chemoattractant protein-1, PFA=paraformaldehyde, LLME=L-leucine-methylester, BrdU=bromodeoxyuridine, TGFa=transforming growth factor-alpha, OTM=olive tail moment, FACS=fluorescence-activated cell sorting, ALD=aldehyde dehydrogenase, NK cells=natural killer cells, CSC=cancer stem cell, IC50=, PDT=photodynamic therapy, PS=photosensitizer, EGFR=epidermal growth factor receptor, AUC=area under the curve, EMMPRIN=extracellular matrix metalloproteinase inducer, ATP=adenosine triphosphate, LDH=lactate dehydrogenase, TUNEL=terminal deoxynucleotidyl transferase dUTP nick end labeling, GnRH-II=gonadotropin-releasing hormone-II, RT-PCR=real time polymerase chain reaction, ATF3=ctivation of transcription factor 3, IHC=immunohistochemistry, RT=radiotherapy, Gy=gray, TNFa=tumor necrosis factor-a, NOS2=nitric oxide synthase 2, STING=stimulator of interferon genes, IFNa=interferon-a, CCL3=chemokine (C-C motif) ligand 3, PARP=poly (ADP-ribose) polymerase, IDO1=indoleamine 2,3-dioxygenase, PD-L1=programmed death-ligand 1.

Chapter 10

Tumor-derived ex-vivo histocultures of head and neck squamous cell carcinoma for the evaluation of DNA double-strand break repair and radiosensitivity

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In preparation

Chapter 11

General discussion

General discussion

Head and neck squamous cell carcinoma (HNSCC) represent a heterogeneous group of tumors, in part explained by different anatomical locations, aetiologies, and a broad range of genetic and molecular changes driving carcinogenesis.[1-3] This heterogeneity leads to several clinical challenges in the prevention and management of HNSCC. For example, tobacco and alcohol directed preventive policies have led to a decrease of the incidence of tobacco and alcohol-related HNSCC while the incidence of HPV-related OPSCC has been increasing worldwide in the last decades. Because this group of HNSCC has different clinical and biological characteristics, new strategies against and increased knowledge about this type of tumors are needed, especially when new (preventive) measures or possibilities for early detection are available, but not widely known.[4,5] Another negative effect of the heterogeneity of HNSCC is that improvement of therapeutic results in difficult. Survival rates of particularly HPV-negative HNSCC patients have hardly increased over the last decades and approximately 50% of patients with advanced disease still develop recurrent disease or metastases.[6] Standard of care, as well as more recently approved new treatment approaches (EGFR and immune checkpoint inhibition) show limited response rates and/or substantial side effects.[7,8] Furthermore, because of the heterogeneity of HNSCC prediction of therapy response is challenging. Therefore, there is an urgent need for biomarkers predicting therapy response for the individual patient, especially when new therapeutic options have been developed.[9,10] This thesis sheds light on these clinical challenges and describes the knowledge of HPV as a risk factor for OPSCC, addresses unanswered questions regarding HPV genome integration, explores the efficacy of novel targeted therapies, and investigates the role of ex-vivo tumor culture models for personalized treatment response prediction.

HPV awareness and viral genome integration

The increasing incidence of HPV-related OPSCC cases worldwide highlights the importance of HPV awareness, specifically the knowledge of HPV as a risk factor for non-uterine cervical cancers. Whereas a national screening program for uterine cervical cancer aims to identify possible lesions in an early stage, precursor lesions for HPV-related OPSCC are scarce and do not seem to follow a stepwise pattern of dysplasia, intraepithelial neoplasia, and invasive malignancy.[11-13] Epidemiological studies demonstrated an association between the presence of HPV DNA in oral rinses/brushes, as well as seropositivity for HPV specific antibodies and the risk for OPSCC.[14-16] However, the low prevalence of this disease hampers clinical utility of these screening approaches and thus no preventive screening tool for the general population has been validated so

far.[12,17] Therefore, large-scale vaccination programmes seem to be the most successful approach for widespread primary prevention of HPV-related malignancies, including OPSCC.[5] In the Netherlands, girls at 13 years of age have been offered an HPV vaccination since 2009. In 2021, this national vaccination program has been extended to include boys and girls form the age of 9. Even though the HPV vaccination rate increased with almost 20% over the past few years, vaccination rate is still suboptimal, indicating missed opportunities in terms of primary prevention. The Netherlands Institute for Public Health and Education (RIVM) reported that 62% of girls and 53% of boys participated in the HPV vaccination program (provisional data of January 2023).[18] Several barriers to HPV vaccination have been identified, including lack of health care provider recommendations, parental concerns, and a general lack of knowledge about HPV (vaccination).[19] This thesis underscores the limited HPV awareness among the Dutch general population (**Chapter 2**). Approximately 31% of study subjects were aware of the existence of HPV, showing higher awareness among women, participants of younger age (18-29 years of age) and participants with higher educational level.[20] The causal relationship between HPV and OPSCC is only recognized by 11% of all subjects. Raising HPV awareness through interventions is crucial to optimize HPV vaccine coverage and ultimately the elimination of HPV-related malignancies. Several studies have shown that interventions, including education, information campaigns, decision support, sending reminders, and facilitating vaccination locations, all increase vaccination coverage by 10-20%.[21] Similarly, vaccine uptake in Ireland increased from 51% to 62% within a year after the establishment of a steering group, setting up focus groups on parental attitudes and engaging in social media.[22] This suggests that there is no single solution to increase HPV awareness and vaccine coverage. Ideally, different approaches should be used for different target populations. In 2023, more than 2 million young adults between 19 and 27 years of age and all unvaccinated adolescents <18 years of age in The Netherlands will receive an invitation for HPV vaccination. The effects on vaccine coverage of this one-time extension of the national vaccination program are awaited next year.

Even though a population-wide screening program for HPV-related OPSCC does not seem feasible at the moment, effective secondary prevention may be achieved by early recognition of the disease by well-informed healthcare providers, including general practitioners (GPs). As the effect of gender-neutral HPV vaccination will be expected in at least 20-30 years from now, vigilance of GPs will remain essential for early detection of HPV-related (pre)malignancies.[23,24] In **Chapter 3**, we observed that a quarter of Dutch GPs lack knowledge on the causative link between HPV and OPSCC.[25] Moreover, we identified knowledge gaps in recognizing patient characteristics, i.e., generally younger

males without a history of intensive smoking and/or alcohol consumption. Further training in the form of regional, national, or virtual meetings may contribute to better targeted knowledge on this relatively rare disease. This is supported by a study showing a significant increase in knowledge of HPV-related OPSCC and the ease to discuss this with patients among GPs, head and neck specialists, and nurses after a 1-hour training session.[26] The results of this thesis have been used for the national 'Make Sense Campaign', a yearly initiative from the Dutch Working Group on Head and Neck Tumors (NWHHT) to raise awareness on HNSCC and the role of HPV. Furthermore, study outcomes of chapter 3 are published in the Dutch journal for GPs 'Huisarts & Wetenschap' (**Chapter 4**).[27] Furthermore, we developed an informative quiz about the importance of HPV infections and vaccination that was shared via social media of the Maastricht University Medical Center on International HPV Awareness Day 2023. Importantly, the increasing incidence of HPV-related OPSCC is a worldwide health problem. Similar studies showed a limited awareness among the population in the USA (36%), the UK (38%), and Germany (17%).[28-30] Whereas awareness among GPs in the UK (74%) and Poland (80%) is comparable to The Netherlands, GPs in Jordan (43%), Germany (54%), and Italy (38%) are less well aware of the link between HPV and OPSCC.[23,31-34] This suggests a cross-border approach and international collaborations to increase HPV awareness and reduce global cancer burden.

Although HPV-related OPSCC generally have a favorable prognosis compared to their HPV-negative counterpart, a subgroup of 10-20% of HPV-positive patients will develop recurrent disease after treatment, resulting in a poor prognosis.[35] Studies have shown that additional risk factors, including older age, smoking, advanced nodal stage, EGFR overexpression, and chromosomal instability contribute to a worse prognosis in HPV-positive OPSCC patients.[35-40] Additionally, a correlation between worse prognosis, deregulated metabolic pathways, and HPV genome integration was observed OPSCC patients.[41] However, studies investigating the relation between HPV integration and patient prognosis have shown inconsistent results so far (**Chapter 5**).[42] On the one hand, this could be attributed to the observed variation in HPV integration patterns, chromosomal locations, and potential consequences for viral and/or human gene expression. On the other hand, inconsistent results between studies could be explained by the use of different detection techniques for HPV integration, which are often biased, insensitive and/or nonspecific, and generally unsuitable for FFPE tissues. Therefore, a novel sequencing approach (FFPE-TLC) to detect HPV integration sites was developed and validated in **Chapter 6**, based on the ligation of DNA sequences, including viral sequences, in close proximity to each other. It was observed that this approach robustly identifies HPV integration sites in HNSCC cell lines as well as FFPE tissues and offers

important advantages over PCR-based detection techniques. The FFPE-TLC method enables sequencing of up to hundreds of kilobases around the integration site, providing maximum information on the flanking (human) sequences, including structural variants, resulting in better and more reliable mapping of these sequences to the human genome. Importantly, this approach allows the assessment of HPV integration in a large study cohort of routinely processed and readily available FFPE material, without the need to collect fresh frozen tumor samples. These adequately powered, unbiased, population-based studies are essential to provide answers to the urgent question on the clinical significance of HPV integration in OPSCC and its value in risk stratification of patients. In addition, the application of FFPE-TLC method could be valuable for clonality assessment of HPV-related tumors, which is currently complicated by the generally low mutational burden and copy number alterations in these tumor type (**Chapter 6**). Ideally, future studies investigating HPV integration should use a uniform NGS-based detection technique, such as the FFPE-TLC approach proposed in this thesis, and integration sites should be validated using another technique to ensure robustness and eliminate experimental or computational artifacts, especially for FFPE tissues.[43]

New therapeutic options and tumor-derived prediction models

Local or regional recurrence or the development of distant metastases after treatment occurs in approximately 20% of patients with early-stage HNSCC and 50% of patients with locally advanced HNSCC and is associated with a poor prognosis.[44-46] Furthermore, conventional therapy, generally consisting of surgery and/or (chemo)radiotherapy, often results in permanent function impairments, of for example chewing, swallowing, and speaking.[47-49] Also, the introduction of immune checkpoint and EGFR inhibitor(s) for the treatment of HNSCC has led to a limited improvement in survival.[2] These challenges in treatment approaches, together with new insights in molecular pathogenesis, led to the introduction of alternative treatment options. On the one hand, antiviral agents (i.e., acyclic nucleoside phosphonates) have been proposed to exhibit anti-proliferative effects, also in tumors without viral origin.[50-52] On the other hand, options for therapies targeting specific genetic alterations and/or deregulated cellular pathways have been extensively explored for the treatment of HNSCC patients.

The antiviral agent cidofovir is currently approved for the treatment of cytomegalovirus retinitis in AIDS patients and used off-label for patients with recurrent respiratory papillomatosis (RRP).[53,54] Moreover, cidofovir was shown to exhibit anti-proliferative properties in cell lines and xenograft mouse models for glioblastoma and nasopharyngeal carcinoma.[50,55,56] In line with this, we observed inhibition of cell growth of HPV-positive and HPV-negative HNSCC cell lines after cidofovir treatment, associated

with the induction of DNA damage, activation of the DNA damage repair machinery, and the occurrence of mitotic catastrophe and cell cycle arrest[57] (**Chapter 7**). However, concerns were raised on the safety of cidofovir, including nephrotoxicity and an increased risk of malignant transformation. For example, a 26-weeks toxicology study in rats revealed an increase in mamma adenocarcinomas after intravenous injection of cidofovir. In addition, some reports describe dysplasia in humans after the use of intralesional cidofovir in RRP.[58] These concerns led to the cessation of off-label cidofovir administration for RRP by many otorhinolaryngologists.[59,60] Consequently, two independent retrospective studies evaluated RRP patients (275 and 154 patients) treated with intralesional cidofovir, and no evidence was found for an increased risk for laryngeal malignancy after cidofovir treatment compared to non-cidofovir treated patients.[61,62] Furthermore, nephrotoxic effects are only observed with high-dose systemic administration of cidofovir. Therefore, intralesional cidofovir treatment should be feasible and this thesis demonstrates effective anti-proliferative properties with mitotic catastrophe as underlying mechanism. Clinical trials are required to provide evidence on the efficacy of cidofovir as a therapeutical agent in (a subset of) HNSCC patients.

Since most genetic alterations in HNSCC occur in tumor suppressor genes and restoring loss of function of these genes remains challenging, options for potentially effective therapies targeting actionable gene mutations are limited. As a consequence, efforts have been focusing on the development of new agents targeting deregulated pathways, including the EGFR, VEGFR, PI3K, c-MET, and CDK4/6 signaling pathways.[63] Cell cycle control genes, including *CCND1*, *CDKN2A*, and *CDK4/6*, are commonly affected in HNSCC, particularly in HPV-negative tumors.[64] In **Chapter 8** of this thesis, the *in-vitro* efficacy of two CDK4/6 inhibitors (palbociclib and ribociclib) was investigated using HPV-positive and HPV-negative HNSCC cell lines. Our results showed that these inhibitors decrease cell proliferation of HPV-negative cell lines specifically, associated with the induction of cell cycle arrest and cell senescence, rather than cell death. These findings are also supported by other preclinical studies, suggesting that CDK4/6 inhibitors should be combined with other therapies to overcome the possible tumor promoting effects of senescent cancer cells.[65-70] In a phase II study with 62 platinum or cetuximab resistant HPV-negative HNSCC patients, palbociclib showed a synergistic effect combined with cetuximab, suggesting a role of palbociclib in overcoming resistance to EGFR-targeted therapy.[71] However, the combination of palbociclib with carboplatin did not result in improved outcomes for patients with recurrent/metastatic HNSCC.[72] The results described in chapter 8 of this thesis demonstrate a synergistic effect on cancer cell

viability when palbociclib is combined with PI3K inhibitor alpelisib, providing another direction for combinational targeted treatment in HNSCC patients.

The PI3K/Akt/mTOR pathway is the most deregulated cancer-driving signaling pathway in HNSCC in both HPV-negative and HPV-positive tumors.[63,73,74] In Chapter 8, we showed that multiple PI3K inhibitors (alpelisib, buparlisib, gedatolisib) effectively decreased cell growth of both HPV-positive and HPV-negative HNSCC cell lines, generally caused by the induction of apoptosis and deregulating cellular metabolism. In line with this, both alpelisib and buparlisib have demonstrated antitumor effects on HNSCC in mouse xenografts.[75,76] Clinical evaluation of PI3K inhibitors in HNSCC is mainly in early phase at this moment. Two phase I studies showed that alpelisib has a manageable safety profile when combined with cisplatin-based chemoradiotherapy or cetuximab plus radiotherapy.[77,78] Similarly, a combination of buparlisib and paclitaxel showed improved progression free survival (PFS) in recurrent/metastatic HNSCC patients who received previous platinum treatment.[79] Based on this study, a phase III trial is currently recruiting almost 500 patients to assess buparlisib efficacy and safety in combination with paclitaxel (NCT04338399). Besides PI3K inhibitors, Akt and mTOR inhibitors have been investigated for the treatment of HNSCC. Both inhibitor types have shown to inhibit tumor cell proliferation and induce apoptosis in cell lines and xenograft models of HNSCC.[80-83] Whereas clinical data on the efficacy of Akt inhibitors are insufficient and early phase trials are ongoing, inhibition of mTOR with temsirolimus showed promising response rates combined with cetuximab in a phase II trial in cetuximab resistant HNSCC patients. However, no improvement of PFS was observed.[84] For the dual PI3K/mTOR inhibitor gedatolisib, combined with palbociclib, a phase I trial is ongoing in advanced solid cancers, including HNSCC (NCT03065062). This thesis provides insights into differences in treatment efficacy and its underlying mechanisms between several PI3K/Akt/mTOR pathway inhibitors, providing the basis for inhibitor selection, decisions on combinational therapies, and the initiation of new clinical trials.

For the translation of new therapeutic options to clinical practice, several aspects need to be considered.[85,86] First, knowledge coming from preclinical in-vitro and animal studies should be translated to and verified in clinical trials. If successful, a positive therapeutic effect, e.g., an increase in overall survival, cannot per definition be translated to the individual patient. As a consequence, reliable biomarkers are essential to predict personalized treatment response. For example, the presence of HPV in OPSCC generally predicts a favorable treatment response, however, a subgroup of HPV-positive OPSCC cancer patients do not show this favorable response and develop local or regional disease or distant metastases. For this group, no specific biomarkers are

available, yet. Also for already approved targeted treatments, objective and durable responses are only reached in a small group of patients. For applied immune checkpoint inhibitors (nivolumab and pembrolizumab) and EGFR inhibitor cetuximab objective responses are only observed in 10-20% of patients.[87-90] These low response rates are most likely attributed to the above-mentioned heterogeneity of HNSCC and pre-existing and acquired resistance mechanisms, resulting in a complex interplay of signaling cascades and the emergence of compensatory pathways.[91]

Patient-derived tumor culture models could offer the possibility to predict therapy response for the individual patient, allowing personalized treatment decision-making in the primary and/or adjuvant setting. In **Chapter 9** of this thesis, available tumor models for HNSCC are summarized and evaluated for their application as a preclinical prediction model for therapy response.[92] A range of preclinical tumor models has been introduced for HNSCC, including primary monolayer cultures, spheroids, organoids, histocultures, xenografts and microdevices. To evaluate the suitability of these culture models for preclinical prediction assays, multiple (technical) aspects were considered, such as culture success percentage, culture duration and complexity, resemblance to the original tumor, and predictive value for patient treatment response. The histoculture model showed the highest success rate and resemblance to the original tumor, as cell-cell interactions and cell-stroma interactions are left intact. In addition, this model allows for a rapid read-out, which is essential for implementation in clinical practice, in contrast to organoid models which typically require a culture period of weeks to months. Interestingly, Runge *et al*. recently revealed that the immune cell compartment remains functional in histocultures, and cytotoxic T-cells could be activated by immuno-stimulatory antibodies, opening doors for the use of histocultures in uncovering actions and predictive biomarkers for immunotherapies.[93] In addition, this model showed to predict patient chemotherapy response with an accuracy of 74-79%.[92] Recent studies focused on the use of histocultures for the evaluation of response to radiotherapy, with the visualization of DNA damage (repair) as a novel read out method.[94-96] One of these studies evaluated the correlation between radiosensitivity in histoculture and corresponding tumor, observing a significantly higher 4Gy/0Gy ratio of residual DNA double strand breaks for patients that responded well to therapy.[96] In **Chapter 10** of this thesis, we implemented the histoculture approach for primary HNSCC and examined its use for the evaluation of DNA damage repair as a measure for radiosensitivity. Good tumor tissue viability in short-term culture as well as differences in DNA damage repair capacity after ex-vivo radiotherapy between patients were observed. Because of the small patient cohort in this study, no direct comparison between ex-vivo response and patient response was possible yet. Nevertheless, this thesis provides the first evidence

on the performance of this model for the evaluation of DNA repair and radiosensitivity and lays the foundation for future studies with larger patient cohorts. Nevertheless, several technical considerations should be taken into account, including the limited reproducibility and the need for a relatively voluminous tumor tissue to test different treatment conditions. Whereas (chemo)radiotherapy is generally the primary treatment choice for most HNSCC in The Netherlands, tumor tissue should be collected from a diagnostic biopsy. This may complicate the use of the histoculture model for very small tumors, including HPV-positive OPSCC, which not rarely present as occult primary lesions.

In the meantime, the organoid model has been increasingly applied for a variety of cancer types, but studies using organoids as a model for HNSCC are still sparse. A single study by Driehuis *et al.*, evaluating the predictive value of HNSCC tumor organoids for radiosensitivity, was included in the literature review of **Chapter 9.**[97] Recently, this group published an extended biobank of HNSCC organoids in which they also explored potential biomarkers for response evaluation.[98] Remarkably, these authors were now able to successfully culture and preserve organoids derived from HPV-positive tumors, which typically grow poorly outside the human body. However, it is not clear what factors contributed to the now successful culture of these HPV-positive organoids. Furthermore, they observed a positive correlation between organoid response (cell viability after irradiation) and response of patients receiving adjuvant radiotherapy (15 patients). However, no correlation was observed between organoid response and clinical response of patients receiving primary radiotherapy, which the authors attribute to the low sample size of only 6 patients.[98] Taken together, the field of ex-vivo culture models for HNSCC is moving forward, but large studies with adequate head-to-head comparisons between ex-vivo response and patient response are still lacking. In addition, efforts should be made to refine culture conditions and develop functional readout methods and clinically relevant predictive biomarkers. Ultimately, these ex-vivo approaches might provide the highly required guidance for personalized treatment of the heterogeneous HNSCC patient population.

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Addendum

Impact paragraph

Impact paragraph

Head and neck squamous cell carcinoma (HNSCC) covers a heterogeneous group of tumors, leading to clinical challenges in the prevention and management of this disease.[1-3] The studies in this thesis have added to our understanding of HPV awareness in The Netherlands, HPV genome integration and its detection, the efficacy and underlying mechanisms of novel targeted therapies, and the application of ex-vivo tumor culture models for the prediction of therapy response. In this impact paragraph, we will place our findings into a scientific and societal perspective.

Awareness is key: implications for primary and secondary prevention

The increasing incidence of HPV-related head and neck cancers highlights the importance of HPV awareness and efforts for effective prevention. The results of this thesis show that the public awareness of HPV and the association of HPV with oropharyngeal cancer is still suboptimal (**Chapter 2**).[4] The identification of these knowledge gaps illustrates the necessity for improvement of HPV awareness, e.g., in the form of (public) education programs and interventions, aiming to increase HPV vaccination coverage and ultimately the elimination of HPV-related malignancies. To raise awareness for HPV-related oropharyngeal cancer in the population, the results of this thesis have been used for the national 'Make Sense Campaign' by the Dutch Working Group on Head and Neck Tumors (NWHHT). In addition, an informative quiz was shared on social media of the Maastricht University Medical Center on International HPV Awareness Day. In November 2023, our study on the lack of HPV awareness in the population has been recognized by the media and results were published in more than 10 regional and national newspapers online and on paper. Amongst others, *NOS Nieuws* published an article entitled "Te weinig mensen weten dat HPV-virus keelkanker kan veroorzaken", describing the results of our study. Similar studies in other European countries and the USA underscore the general lack of HPV awareness and further discussions are currently ongoing to organize cross-border collaborations to increase HPV awareness and reduce global cancer burden.

Well-informed healthcare providers, such as general practitioners (GPs), also play an important role in the stimulation of vaccine uptake for both boys and girls. Our results in **Chapter 3** demonstrate that the knowledge of the link between HPV and oropharyngeal cancer as well as patient characteristics among GPs in The Netherlands offer room for improvement.[5] Awareness of HPV-related head and neck cancer and corresponding patient characteristics among GPs would lead to early detection of these tumors, timely treatment, and improved patient outcome. Therefore, GPs that participated in our study

received a fact sheet on HPV and its role in oropharyngeal cancer and our findings on HPV awareness among GPs has been shared with this occupational group via the Dutch Journal for GPs 'Huisarts & Wetenschap' (**Chapter 4**).

Methodological development: implications for future studies into HPV integration

Besides new discoveries, progress in science also relies on the development, refinement, and validation of methodologies. Although HPV genome integration is a common genetic event in HPV-related oropharyngeal tumors, its biological consequences for disease progression and patient prognosis are still unclear. Importantly, limitations of current HPV integration detection methods have hampered research into the clinical relevance of HPV integration (**Chapter 5**).[6] This thesis describes a novel sequencing-based approach that enables sequencing of longer DNA sequences, which is especially valuable for formalin-fixed, paraffin-embedded tumor tissue. The proposed method will enable the assessment of HPV integration in a large study cohort of readily available tumor material, without the need for the collection of fresh frozen tumor tissue. Together with mRNA expression profiling, application of this method will lead to a better understanding of the causes and consequences of HPV integration and the identification of prognostic and/or predictive biomarkers for patients with HPV-related oropharyngeal cancer. Once validated, such biomarkers may play a significant role in patient stratification and the choice for treatment modality. In addition, the presence of HPV integration, including exact integration number and integration location, could guide clonality assessment of multiple tumors with HPV-involvement in one patient, with therapeutical implications. Lastly, the proposed sequencing approach may be applied for integration detection of other (oncogenic) virus types, or additional indications in which the sequencing of long DNA sequences is required, such as the identification of chromosomal rearrangements or gene fusions in multiple cancer types.

Turning knowledge into personal benefit: implications for therapeutic strategies

The general aim of the studies performed in this thesis is to turn knowledge into a benefit for society but also for the individual patient. Over recent decades, survival rates for HNSCC patients have hardly increased and current treatment-related side effects are still substantial. The identification of novel targeted therapies offers a promising direction towards an improved, more personalized treatment approach. In **Chapter 7** and **Chapter 8**, we show that the antiviral agent cidofovir and multiple CDK4/6 and PI3K/Akt/mTOR pathway inhibitors effectively reduce HNSCC cell proliferation. The

obtained knowledge on their efficacy and underlying molecular mechanisms serves as a foundation for future (pre)clinical studies, investigating the value of these agents, as monotherapy or in combination with existing treatment modalities, in the targeted treatment of HNSCC patients.

The use of ex-vivo tumor-derived culture models for treatment response prediction has gained much attention over recent years. In this thesis, we aimed to summarize the variety of proposed culture models to provide a comprehensive and structured overview of advantages, disadvantages, nomenclature, and possible applications as a reference point for (new) investigations into the predictive value of these model systems (Chapter 9). In addition, we developed and validated a histoculture model for the evaluation of radiosensivity and observed varying response rates between individual patients (Chapter 10). Once validated in a large study with adequate comparison to clinical patient data, this assay could be a valuable tool for the personalized selection of existing treatment modalities as well as testing of novel therapeutic options. Next to head and neck cancer, this application could easily be adopted for other tumor types and (DNA damaging) therapies.

The results in this thesis present new opportunities to decrease the impact of head and neck cancer by serving as a basis for (pre)clinical research and by contributing to new developments in prevention and personalized treatment of head and neck cancer patients. Our findings have been communicated on various (inter)national conferences and are published or will be published in peer-reviewed, preferably open access journals. Thereby, the created knowledge is made available to experts in the field and serves as a new starting point for future research.

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Addendum

Summary / Samenvatting

Summary

Head and neck squamous cell carcinoma (HNSCC) is the most common type of head and neck cancer (95% of cases) and represents 5% of all cancer diagnoses worldwide with an overall mortality rate of approximately 50%. HNSCC covers a heterogeneous group of tumors, leading to several clinical challenges in its prevention and management, including increasing incidence of HPV-related tumors, high recurrence rates, substantial treatment-related side effects, and difficulties to predict treatment efficacy for individual patients. This thesis aimed to 1) assess and increase HPV awareness among the population and general practitioners (GPs) in The Netherlands with the goal to increase HPV vaccination coverage and stimulate early detection; 2) improve the knowledge on HPV genome integration and associated detection techniques in order to identify prognostic biomarkers; 3) investigate potential new targeted therapies to improve treatment options; and 4) explore the suitability and applications of tumor-derived culture models to guide personalized treatment for HNSCC patients (Chapter 1).

While the incidence of tobacco related HNSCC has declined in the past two decades, there is an increase in HPV-related oropharyngeal cancer (OPSCC) cases in Western countries. In contrast to patients with non-HPV-related OPSCC, patients with HPV-related OPSCC are generally younger, more often male, have a higher socioeconomic status, and are less likely to have a history of extensive tobacco and alcohol use. Knowledge about HPV and the association of HPV and OPSCC, both in the general population and among health care professionals, will promote HPV vaccination coverage, early detection of the disease, and ultimately patient outcome. In Chapter 2, the current knowledge on HPV and HPV-related OPSCC among the general population in The Netherlands was explored using a cross-sectional survey study. Our data revealed that 30.6% of the participants (N=1044) had heard of HPV. This awareness was significantly lower in males, participants older than 65 years of age, participants with low educational level and current smokers. Of the participants who had heard of HPV, 29.2% was aware of the causative link between HPV and OPSCC (11% of total population). In addition, almost 50% of participants knew about the existence of an HPV vaccine. Our findings indicate that targeted knowledge in the Dutch population is lacking and increasing the awareness of HPV is required to improve vaccination coverage and (primary) prevention. In this context, the results of this thesis have been used for the national 'Make Sense Campaign' by the Dutch Working Group on Head and Neck Tumors (NWHHT).

The HPV awareness among GPs in The Netherlands was assessed in Chapter 3. A total of 207 GPs throughout The Netherlands participated in our cross-sectional study, of which 72% recognized HPV as a risk factor for OPSCC and 76.3% was aware of the increasing incidence rates. Patient characteristics were less well recognized, with 35.7% of GPs knowing that patients with HPV-related OPSCC are more often male and just over half was aware of the younger age of patients. Our findings demonstrate that GPs are reasonably aware of HPV as a causative factor for OPSCC, however, a quarter of GPs is still unaware of this link. Specific knowledge on patient characteristics could be improved, to ensure early recognition of the disease in this relatively young patient group without classical risk factors. Further training in the form of regional and national meetings may contribute to better targeted knowledge and appropriate referral of patients to secondary care. To raise HPV awareness among GPs in The Netherlands, findings described in this thesis were summarized in an infographic and published in the Dutch Journal for GPs 'Huisarts en Wetenschap' (Chapter 4).

Despite the generally better prognosis of HPV-related OPSCC compared to their HPV-negative counterpart, a still unidentified subgroup of 10-20% of HPV-positive patients will develop recurrent disease after treatment. Integration of the HPV genome in the human genome has been suggested to have further reaching consequences for tumorigenesis of HPV-positive tumors, but studies on the relation between HPV integration and patient outcome have shown inconsistent results. In Chapter 5, we summarized the recent literature on HPV integration in OPSCC and observed that HPV integration prefers less protected and more accessible chromosomal regions, including highly transcribed (cancer) genes. In addition, several factors were described to promote viral integration, including reactive oxygen species, inflammation, and APOBEC expression. HPV integration could lead to constitutive expression of viral oncogenes and deregulation of cellular (cancer) genes, possibly conferring neoplastic pressure. Importantly, different techniques to detect HPV integration have been used in studies so far, which are often biased, insensitive and/or nonspecific. Together with the variety of described bioinformatic approaches for integration detection, this might explain the inconsistent data on integration percentage and clinical relevance. Furthermore, current detection techniques are generally unsuitable for formalin-fixed, paraffin-embedded (FFPE) tumor tissues. Therefore, in Chapter 6, we developed and validated a novel sequencing approach (cell-based TLA, FFPE-TLC), based on the ligation of DNA sequences in close proximity to each other. Seven HPV-positive cell lines and FFPE tissues of 27 HPV-positive OPSCC were used for HPV integration detection using this method. Our findings demonstrate that this approach enabled sequencing of hundreds of kb around the HPV integration site, detecting exact HPV integration loci, structural

variants, and chromosomal rearrangements in both HPV-positive cell lines and FFPE tissues of OPSCC. HPV integration sites were detected in 15/27 FFPE tumor tissues and confirmed by PCR analysis and Sanger sequencing in a subset of samples, showing simple and complex integration patterns, resulting in structural variations, which also may explain mechanisms underlying integration and clonal evolution. This FFPE-TLC method provides the opportunity for reliable and robust detection of HPV integration sites, specifically valuable for FFPE tissues, enabling further research on the clinical relevance of HPV integration in OPSCC, including clonality assessment of multiple tumors with HPV-involvement within a patient.

Over the last decades, survival rates for HNSCC patients have hardly increased, recurrence rates are still high, especially for HPV-negative HNSCC, and treatment-related side effects are substantial. Therefore, there is an urgent need for improved, more targeted treatment options. In **Chapter 7**, the antiproliferative effect of the antiviral agent cidofovir was investigated using HPV-positive and HPV-negative HNSCC cell lines. It was observed that cidofovir treatment resulted in inhibition of cell growth in all cell lines, and that DNA damage accumulated and activated the DNA damage pathway, leading to G2/M cell cycle arrest. Moreover, our findings suggest the occurrence of mitotic catastrophe, without the induction of apoptosis.

In addition to antiviral therapies, options for therapies targeting specific genetic alterations and/or deregulated cellular pathways have been a field of interest for the treatment of HNSCC patients. Cell cycle control genes, as well as the PI3K/Akt/mTOR pathway are commonly affected in HNSCC. In **Chapter 8**, the therapeutical efficacy of several CDK4/6 (palbociclib, ribociclib) and PI3K/Akt/mTOR pathway inhibitors (alpelisib, buparlisib, gedatolisib) was explored *in-vitro*. Both inhibitor types showed to inhibit their respective pathways and cell growth. The CDK4/6 inhibitors showed to be mainly effective in HPV-negative HNSCC cell lines, inducing cell cycle arrest in G1 phase without the induction of apoptosis. Treatment with PI3K/Akt/mTOR inhibitors resulted in inhibition of cell proliferation of both HPV-negative and HPV-positive HNSCC cell lines, the induction of apoptosis, and the attenuation of oxidative and glycolytic cellular metabolism. Furthermore, we observed that the combination of a CDK4/6 and a PI3K/Akt/mTOR inhibitor, i.e., ribociclib and alpelisib, synergistically decrease cell viability. Further research should elucidate whether (a combination of) these inhibitors are effective therapeutic agents for HNSCC patients.

Due to the heterogeneous nature of HNSCC, varying response rates to both standard-of-care and new treatments are observed between patients. Tumor-derived culture models

offer the chance to predict therapy response in a personalized setting. In Chapter 9, we summarized available culture models for HNSCC, and evaluated their application as a preclinical prediction model for therapy response. Results demonstrate that a wide range of primary culture models has been introduced for HNSCC, including monolayer cultures, spheroids, organoids, histocultures, xenografts, and microdevices. Technical aspects of these culture models were assessed, such as culture success percentage, culture duration and complexity, resemblance to the original tumor, and predictive value for patient therapy response. The histoculture model was most often used, and showed the best success rate, tumor resemblance, and prediction of patient response to chemotherapy with ~75% accuracy. Although radiotherapy, either or not in combination with chemotherapy, is an important treatment modality for HNSCC, evidence on the use of histocultures to predict radiosensitivity is limited. In Chapter 10, we aimed to characterize a short-term HNSCC histoculture model, derived from fresh tumor tissue, and evaluated its application to study repair capacity of irradiation-induced DNA damage as a measure for radiosensitivity. During short-term culture (48h), histocultures maintained tissue architecture, including different cell types, epithelial marker expression, and tumor cell proliferation. Cultures derived from HPV-positive tumors maintained their strong expression of the HPV surrogate maker p16. Ex-vivo irradiation of histocultures resulted in increased DNA double strand breaks, visualized by 53BP1 foci, and differences in repair capacity, assessed at 24 hours after irradiation, were observed between individual patients. Specifically, cultures derived from HPV-positive tumors showed significantly less efficient DNA repair. The combination of cisplatin and radiotherapy decreased DNA repair efficacy in 3 out of 4 tumors compared to radiotherapy alone. Sufficiently powered studies, with adequate comparison between ex-vivo response and patient response are required to elucidate whether histocultures can mature into useful clinical tools.

Finally, in Chapter 11, the results described in this thesis are discussed and reflected in the light of current knowledge in the field. The new insights presented in this thesis might provide new opportunities to decrease the impact of HNSCC and open doors for future research.

Samenvatting

Hoofd-halskanker, met het plaveiselcelcarcinoom als de meest voorkomende vorm, beslaat ongeveer 5% van alle kankerdiagnoses wereldwijd met een gemiddeld sterftepercentage van 50%. Plaveiselcelcarcinomen in het hoofd-halsgebied vormen een heterogene groep tumoren, met daarbij behorende klinische uitdagingen op het gebied van preventie en behandeling. Ten eerste is er een significante stijging van het aantal humaan papillomavirus (HPV)-gerelateerde keelholtetumoren. Ten tweede is het recidiefpercentage hoog en laten huidige behandelopties vaak ernstige bijwerkingen zien. Daarnaast is het een uitdaging om de meest effectieve behandeling voor elke individuele patiënt te selecteren. Het doel van dit proefschrift was 1) om de kennis over HPV en keelholtekanker onder de Nederlandse bevolking en huisartsen in kaart te brengen en te verbeteren; 2) om meer kennis te genereren over de integratie van het HPV genoom in het humane genoom en de technieken die kunnen worden gebruikt om HPV integratie te detecteren; 3) om nieuwe doelgerichte therapieën te onderzoeken om de behandeling van hoofd-halskanker te verbeteren, en 4) om de geschiktheid en toepassing van tumorkweekmodellen te onderzoeken voor gepersonaliseerde therapieselectie.

Hoewel de laatste twee decennia de incidentie van het aantal hoofd-halstumoren veroorzaakt door roken en alcoholgebruik is afgenomen, is er een wereldwijde stijging in het aantal HPV-gerelateerde keelholtetumoren. Patiënten met HPV-gerelateerde keelholtetumoren zijn vaak jonger, vaker van het mannelijk geslacht, hebben een hogere socio-economische status, roken vaak minder en drinken minder alcohol. Voldoende kennis over HPV en de relatie met keelholtekanker in de Nederlandse bevolking en onder zorgprofessionals zal bijdragen aan een hogere HPV-vaccinatiegraad, vroegere herkenning van de ziekte en uiteindelijk een betere uitkomst voor patiënten. In Hoofdstuk 2 hebben we de kennis over HPV en HPV-gerelateerde keelholtekanker onder de Nederlandse bevolking in kaart gebracht met een vragenlijststudie. Uit de resultaten is gebleken dat 30,6% van alle deelnemers (totaal 1044) nog niet eerder van HPV gehoord had. Deze kennis over HPV was lager bij mannen, deelnemers ouder dan 65 jaar, deelnemers met een lager opleidingsniveau en deelnemers die roken. Van alle deelnemers die van HPV hadden gehoord, wist maar 29,2% dat HPV ook keelholtekanker kan veroorzaken (11% van alle deelnemers in de studie). Bijna de helft van de deelnemers wist dat er een vaccinatie bestaat tegen HPV. Met deze studie hebben wij laten zien dat de kennis over HPV, HPV-gerelateerde keelholtekanker en HPV-vaccinatie onder de Nederlandse bevolking (nog) niet optimaal is en dat het verhogen van deze

kennis belangrijk is om een hogere vaccinatiegraad en daarmee een betere (primaire) preventie te bereiken.

De kennis over HPV onder Nederlandse huisartsen hebben we onderzocht in Hoofdstuk 3. In totaal hebben 207 huisartsen door heel Nederland meegedaan aan onze vragenlijststudie. 72% wist dat HPV keelholtekanker kan veroorzaken en 76,3% bleek op de hoogte te zijn van de stijgende incidentie van HPV-gerelateerde keelholtekanker. Echter, karakteristieken van patiënten met deze tumoren worden minder goed herkend door huisartsen; 35,7% wist dat deze patiënten vaker mannelijk zijn en iets meer dan de helft was op de hoogte van de vaak jongere leeftijd. Deze resultaten laten zien dat een kwart van de Nederlandse huisartsen niet op de hoogte is van de relatie tussen HPV en keelholtekanker en dat met name de kennis over patiëntkarakteristieken verbeterd dient te worden om deze ziekte snel te kunnen herkennen. Bijscholing, in de vorm van regionale, nationale of virtuele bijeenkomsten, zal bijdragen aan een betere kennis en een meer gerichte ziekteherkenning en doorverwijzing van patiënten.

Ondanks dat patiënten met HPV-gerelateerde keelholtekanker vaak een betere prognose hebben dan patiënten met HPV-negatieve tumoren, ontwikkelen 10-20% van hen terugkeer van ziekte na de behandeling (recidief), die niet op voorhand geïdentificeerd kunnen worden. Er wordt gesuggereerd dat de integratie van het HPV-genoom in het humane genoom consequenties kan hebben voor de ontwikkeling en progressie van HPV-gerelateerde tumoren, maar studies die het verband tussen HPV-integratie en patiëntoverleving vergeleken hebben laten inconsistente resultaten zien. In Hoofdstuk 5 van dit proefschrift hebben wij de meest recente literatuur over HPV-integratie in hoofd-halskanker samengevat. Onze resultaten laten zien dat er een voorkeur bestaat voor het HPV-genoom om te integreren in toegankelijke chromosomale gebieden, zoals in (kanker)genen die een verhoogde genexpressie hebben. Daarnaast worden er verschillende factoren beschreven die HPV-integratie kunnen bevorderen zoals zuurstofradicalen, ontsteking of expressie van antivirale eiwitten (APOBEC). HPV-integratie kan leiden tot een continue expressie van virale oncogenen en een deregulatie van cellulaire (kanker)genen, mogelijk met activatie van de tumorgroei tot gevolg. Een andere belangrijke bevinding was dat studies gebruik maakten van verschillende technieken om HPV-integratie te detecteren. Deze technieken zijn vaak niet gevoelig of specifiek genoeg, danwel niet toepasbaar op formaline gefixeerde, paraffine ingebedde (FFPE) tumorweefsels. Daarom hebben wij in Hoofdstuk 6 een nieuwe methode ontwikkeld en gevalideerd om HPV-integratie te detecteren in FFPE weefsels van keelholtetumoren. Deze methode is gebaseerd op het identificeren van DNA-sequenties die bij elkaar in de buurt liggen. Hiervoor hebben wij 7

HPV-positieve cellijnen en FFPE-weefsels van 27 HPV-positieve keelholtetumoren gebruikt. Onze bevindingen lieten zien dat deze nieuwe methode, ook wel FFPE-TLC genoemd, in staat is om grote stukken humaan DNA, alsook veranderingen daarin, in kaart te brengen rondom de virale integratielocus. HPV-integratie werd gevonden in 15/27 keelholtetumoren, waarbij er simpele en complexe integratiepatronen werden gezien. Concluderend hebben we laten zien dat deze FFPE-TLC methode op een betrouwbare en robuuste manier HPV-integratie kan detecteren, waardoor nu prospectief, populatie-gebaseerd onderzoek naar de klinische relevantie van HPV-integratie in keelholtekanker mogelijk wordt. Daarnaast kan deze methode ook een belangrijke toevoeging zijn bij het bepalen van een mogelijke gedeelde oorsprong van meerdere HPV-positieve tumoren in dezelfde patiënt.

Ondanks verbeteringen in de zorg, is de overleving van hoofd-halskankerpatiënten de afgelopen twee decennia nauwelijks verbeterd, blijft het recidiefpercentage, met name voor HPV-negatieve tumoren, hoog en zijn bijwerkingen van huidige medicijnen nog steeds substantieel. Daarom zijn er dringend nieuwe, meer doelgerichte therapieën nodig. In Hoofdstuk 7 hebben we de effectiviteit van het antivirale middel cidofovir getest, waarbij HPV-positieve en HPV-negatieve hoofd-halskanker cellijnen zijn gebruikt. De resultaten van dit onderzoek lieten zien dat cidofovir behandeling groeiremming induceert in de cellijnen, als gevolg van het induceren van DNA-schade, activatie van het DNA-schade reparatiemechanisme en het stilleggen van de celcyclus in de G2/M fase. Bovendien resulteerde cidofovir behandeling in het optreden van afwijkende celdelingen, mitotische catastrofe genaamd, zonder dat apoptose (geprogrammeerde celdood) optrad.

Naast deze antivirale therapie is er de laatste jaren ook veel aandacht voor nieuwe medicijnen die gericht zijn tegen specifieke genetische veranderingen of gedereguleerde processen in (hoofd-hals)kanker. Genen die de celdeling controleren, of een rol spelen in de PI3K/Akt/mTOR signaleringsroute, zijn vaak ontregeld in hoofd-halskanker. In Hoofdstuk 8 van dit proefschrift hebben we met bovengenoemde cellijnen ook onderzocht of verschillende CDK4/6 remmers (palbociclib en ribociclib) en PI3K/Akt/mTOR remmers (alpelisib, buparlisib en gedatolisib) gebruikt kunnen worden om de celdeling te onderdrukken. De CDK4/6 remmers waren met name effectief in HPV-negatieve cellijnen, waarbij ze zorgden voor een blokkade van de celcyclus. Behandeling met de PI3K/Akt/mTOR remmers zorgde voor een afname van celgroei van zowel HPV-positieve als HPV-negatieve cellijnen, de inductie van apoptose, en een verlaging van het celmetabolisme. Daarnaast zorgde een combinatie van ribociclib met alpelsib voor een versterkend remmend effect op de celgroei. Toekomstig onderzoek

moet aantonen of deze remmers gebruikt kunnen worden voor de behandeling van hoofd-halskanker.

De grote heterogeniteit van hoofd-halskanker zorgt ook voor grote verschillen in therapierespons tussen patiënten. Primaire tumorkweekmodellen, direct afkomstig van de tumor, bieden de mogelijkheid om voor de individuele patiënt de gevoeligheid voor bestaande als ook nieuwe medicatie te testen. In Hoofdstuk 9 hebben we in de literatuur beschreven kweekmodellen voor hoofd-halskanker met elkaar vergeleken en onderzocht wat hun waarde is bij het voorspellen van therapiegevoeligheid van de tumor waarvan ze afkomstig zijn. Er zijn veel verschillende soorten kweekmodellen beschreven, zoals primaire cellijnen, sferoïden, organoïden, primair intact weefsel, muis xenograft modellen, en tumor-op-een-chip modellen. Van al deze kweekmodellen werden verschillende technische aspecten beoordeeld, zoals kweek succespercentage, duur en complexiteit van het kweken, de gelijkenis met de originele tumor, en de betrouwbaarheid waarmee therapiegevoeligheid voor de patiënt kan worden voorspeld. Er kon worden geconcludeerd dat voor hoofd-halskanker het kweken van intact weefsel het meest onderzocht was, de meeste gelijkenissen vertoonde met de originele tumor, en de beste voorspellende waarde had voor de gevoeligheid van de patiënt voor chemotherapie (accuraatheid van 75%). Hoewel radiotherapie, al dan niet in combinatie met chemotherapie, de meest toegepaste behandeling is voor hoofd-halskanker, is het gebruik van tumorkweekmodellen voor het evalueren van radiotherapiegevoeligheid nog niet veel onderzocht. Daarom hebben wij in Hoofdstuk 10 van dit proefschrift een model voor het kweken van intact hoofd-halstumorweefsel opgezet, en onderzocht of we dit model kunnen gebruiken voor het analyseren van reparatie van DNA-schade veroorzaakt door bestraling, als maat voor radiotherapiegevoeligheid. De tumorkweken behielden de weefselstructuur van de originele tumor, bestaande uit verschillende soorten cellen, over een kweekperiode van ten minste 48 uur. Daarnaast bleven de expressie van een epitheelcel-marker aanwezig in het tumorweefsel en bleven de tumorcellen delen tijdens de kweekperiode. Bestraling van de tumorkweekmodellen zorgde voor DNA-schade, zichtbaar gemaakt met een kleuring voor 53BP1, een eiwit betrokken bij DNA-schade reparatie. Tussen verschillende tumoren werd er een verschil gezien in de capaciteit om de geïnduceerde DNA-schade te herstellen binnen 24 uur. Met name tumorkweken van HPV-positieve tumoren waren significant minder goed in staat om de DNA-schade efficiënt te repareren. De combinatie van chemotherapie (cisplatine) met radiotherapie zorgde voor een minder efficiënte reparatie van de DNA-schade in vergelijking met radiotherapie alleen. Toekomstige studies met een groter aantal patiënten, waarin therapiegevoeligheid van de tumorkweken en de respons van

de patiënt met elkaar worden vergeleken, zijn nodig om de uiteindelijke klinische waarde van deze testen te bepalen.

In **hoofdstuk 11** worden alle bevindingen in dit proefschrift bediscussieerd en bekeken in het licht van de huidige kennis en literatuur. De inzichten beschreven in dit proefschrift kunnen bijdragen aan nieuwe handvatten om de impact van hoofd-halskanker te verlagen en deuren te openen voor toekomstig onderzoek.

Addendum

Curriculum Vitae

Curriculum Vitae

Imke Demers was born on November 22nd, 1993, in Kerkrade, The Netherlands, as a daughter to Marcel and Marilda Demers and sister to Karlijn and Lotte. In 2012, she graduated from secondary school at Sintermeerten College, Heerlen. She decided to follow her fascination for biology and completed her bachelor's degree in biomedical sciences at Maastricht University in 2015, with a specialization in molecular life sciences (cum laude). In 2017, she obtained her master's degree in biomedical sciences, with a specialization in oncology and developmental biology, at Maastricht University. In 2018, she started as a PhD candidate at the Departments of Pathology and Otorhinolaryngology, Head and Neck Surgery at Maastricht University Medical Center (MUMC+), supervised by Prof. dr. Speel and Prof. dr. Kremer. Her PhD project focused on clinical challenges in head and neck cancer. During her PhD, she worked as a visiting researcher at the University Hospital Heidelberg, Germany, for one month to get familiar with ex-vivo tumor culture techniques and infrastructure. Her work has been honored with a stipendium for the René Vogels Foundation, and a short-list nomination for the Beter Horen Science prize for the best scientific publication in the field of otorhinolaryngology by young researchers. In addition, she received the award for the best oral presentation at the 4th International Symposium for HPV infections in Head and Neck Cancer (Amsterdam, The Netherlands).

Currently, Imke will pursue her career as a clinical molecular biologist in pathology at MUMC+ and Jeroen Bosch Hospital ('s-Hertogenbosch, The Netherlands) as a trainee of Prof. dr. Speel and dr. van den Brule. Furthermore, she will continue her academic career as a postdoctoral researcher in the head and neck cancer field.

Addendum

List of publications

List of publications

Published articles related to this thesis

Demers I, Verhees F, Schouten L, Muris JW, Kremer B, Speel E-JM. Orofarynxkanker veroorzaakt door het humaan papillomavirus. Huisarts Wet 65, 16–17 (2022).

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* Authors contributed equally

Addendum

Dankwoord

Dankwoord

Daar is 'ie dan, het eindresultaat van een bijzondere periode! Hoewel een promotieonderzoek zonder hobbels niet bestaat, kijk ik met een heel goed gevoel terug op de afgelopen jaren. Ik mag me gelukkig prijzen met alle mensen om mij heen die me hebben bijgestaan in het overwinnen van hobbels, hebben meegevierd bij successen en mijn promotietraject nog leuker hebben gemaakt.

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