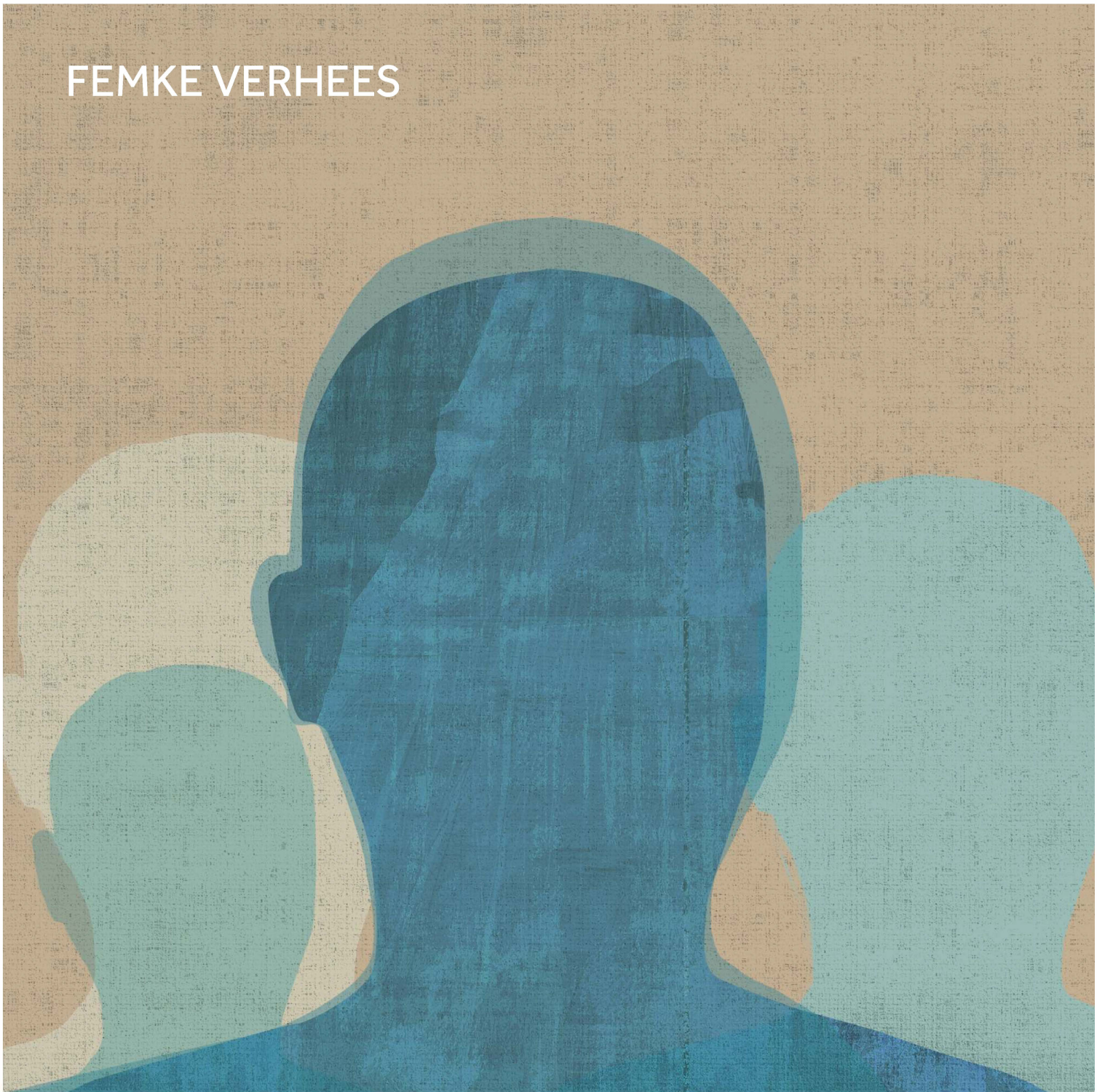
Increasing awareness and therapeutical options to improve prognosis of HPV positive and HPV negative head and neck cancer

INCREASING AWARENESS AND THERAPEUTICAL OPTIONS TO IMPROVE PROGNOSIS OF HPV POSITIVE AND HPV NEGATIVE HEAD AND NECK CANCER

FEMKE VERHEES



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Chapter 1

General introduction

1.1 Head and neck squamous cell carcinoma (HNSCC)

Head and neck squamous cell carcinoma (HNSCC) was the seventh most common cancer worldwide in 2018, accounting for 3% of all cancers.[1] HNSCC encompasses a heterogeneous group of malignancies that predominantly arise from the mucosal surface of the oral cavity, larynx and pharynx and are mainly associated with tobacco and alcohol consumption. In oropharyngeal cancer human papillomavirus (HPV) is also involved as a risk factor in an increasing percentage of patients. A meta-analysis including 5396 oropharyngeal squamous cell carcinomas (OPSCC) observed an increase in prevalence of HPV in OPSCC from 40.5% before 2000 to 72.2% after 2005, including significant increases in North America and Europe.[2] In the Netherlands, an increase in the prevalence of HPV in OPSCC was observed from 5.1% in 1990 to 29% in 2010 in the VU Medical Centre.[3] In Maastricht University Medical Centre there was increase in prevalence from 21.4% in 2003 to 50% in 2011.[4] For UMC Groningen, this increase was from 13% in 2004 to 30% in 2012.[5] Of the many subtypes of HPV, the most common carcinogenic subtype is HPV16, accounting for 80-90% of HPV positive HNSCC.[6] While the number of tobacco-related cancers has declined in the past two decades, there is an increase in HPV-associated oropharyngeal squamous cell carcinomas (OPSCC).[7,8] The incidence of HPV associated HNSCC has now surpassed the incidence of HPV-induced cervical cancers.[9,10]

1.2 Human papillomavirus (HPV)

HPVs are non-enveloped, double-stranded circular DNA viruses that infect the basal cells of cutaneous and mucosal epithelia.[11] A subgroup of 15 HPV types is linked to the development of malignant lesions, i.e. high-risk (HR) HPVs. The circular HPV DNA contains 7 open reading frames for 7 early (E1-E7) and 2 late (L1-L2) proteins. The early (E) region proteins are necessary for viral replication; the late (L) region proteins are required for virion assembly.[12,13] HPV infection is initiated by binding of the virion L1 and L2 protein to heparan sulphate proteoglycans (HSPG) on segments of the basement membrane of disrupted epithelium, which are exposed at sites of micro injury. 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.[14] Earlier studies indicate that the expression of viral E6 and E7 genes contributes to the malignant phenotype of HPV-associated cancers.[15] E6 protein interacts with p53 and targets it to ubiquitination and proteasomal degradation in HPV infected cells. As a result, p53 driven

inhibition of cell cycle arrest and apoptosis is abolished in HPV infected cells. The E7 protein interacts with the unphosphorylated retinoblastoma tumor suppressor (Rb1) protein and targets it to proteasomal degradation, which enables activation of transcription factor E2F leading to transcription of S-phase genes required for progression of the cell cycle. E7-directed Rb1 degradation also promotes overexpression of p16, which is a reliable surrogate marker for HPV infection.[16]

1.3 HPV vaccine

Several prophylactic vaccines including Cervarix, Gardasil® and Gardasil®9 has been approved by the FDA to protect from HPV infection as well as HPV-associated diseases as genital warts and cancer.[17-19] The vaccines protect against HPV types 16 and 18 and the Gardasil vaccines also protects against types 6 and 11. As already mentioned, recent data suggest that the incidence of HPV related OPSCC exceeds the incidence of HPV related cervical cancer.[9,10] The HPV vaccine not only protects against the development of cervical cancer, but also against oropharyngeal cancer.[20] 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.[21] The national vaccination coverage for HPV for girls was 53% in the Netherlands in 2019.[21] The vaccine has been included in the vaccination program for boys since 2021. Since vaccination against HPV became available, awareness of HPV and the association with cervical cancer has dramatically increased in for example the UK, US and Australia.[22] However, how the knowledge is about the association between HPV and oropharyngeal cancer among the general population as well as for example among health care professionals is not that clear yet. A recent study by Lechner et al examined this knowledge under the English population, but there are no similar studies in the Netherlands.[23] In order to maximize the potential benefits of HPV vaccination, it is necessary to get the vaccination coverage as high as possible and therefore it is important to increase awareness of the human papilloma virus, virus-associated cancer and the role of vaccination.

1.4 Prognosis of HNSCC patients

Advances in surgery and radiotherapy as well as the use of multidisciplinary treatment modalities have improved cure rates for locally advanced HNSCC patients.[24] However, overall mortality rates of HNSCC have hardly decreased over the last decades and the five-year survival rate still ranges between 40–50%.[25] Locoregional recurrence,

metastasis and drug resistances are common problems among all HNSCC patients. Most recurrent and/or metastatic HNSCC patients still have a poor survival. To improve response rates and survival, multiple trials are ongoing to evaluate (combination) treatments involving for example immune checkpoint inhibitors, therapeutic vaccines and other targeted agents.

The HPV status of the tumor possesses powerful prognostic value, where HPV positive patients have a more favorable prognosis, due to better treatment response, and lower risks of recurrence and secondary primary tumors.[10,26,27] However, there is a subgroup of HPV positive patients having a less favorable prognosis with a greater risk of recurrence or developing a second primary tumor and active vasculature invasion.[28,29] The far more favorable outcome and especially the reduced prognostic value of lymphatic metastasis of HPV positive OPSCC when compared with HPV negative OPSCC is so substantial that the tumor-node-metastases (TNM) staging for HNSCC was adapted in the eight edition and p16 immunostaining was included as a surrogate marker for HPV status.[30-32] The use of HPV status as a predictive biomarker for dose de-escalation or changing of treatment modalities has been considered, but the value of this approach has not been proven yet.[33]

For uterine cervical carcinomas, it has been shown that integration of viral HPV DNA into the host genome directly correlates to the progression from dysplastic lesions to carcinomas in most of the cases.[34,35] Integration of HPV DNA appears to be a direct result of chromosomal integrity destabilizing processes mediated by the expression of the viral oncogenes E6 and E7. It has already been shown that HPV may also integrate in the genome of HNSCC[36,37], but in previous work no evidence was found that HPV integration significantly affects viral gene expression or the expression of disrupted human genes as compared to tumors with episomal virus.[38-40] So, how HPV integration affects its host cell and whether HPV integration contributes to the prognosis in HNSCC is still unclear.

1.5 Treatment of HNSCC

Current treatment selection in HNSCC patients relies on clinical, histopathologic and radiologic parameters to determine the stage of the disease using the TNM classification. Early stage disease is mostly treated with radiation or surgery alone; locoregionally advanced disease is usually treated with combined approaches including surgery, radiotherapy, and chemotherapy.[41] Combination of radiotherapy with cisplatin remains the standard for inoperable advanced stages. Cetuximab, a monoclonal antibody directed against Epidermal Growth Factor Receptor (EGFR) inhibiting

downstream signaling, is a possible alternative to chemotherapy in patients unfit for cisplatin. For patients with recurrent or metastatic HNSCC novel immunotherapies are an option, including Nivolumab or Pembroluzimab. All treatment modalities severely reduce quality of life. Although immunotherapy has shown durable responses, this benefit is seen in only a limited number of patients. Research into new therapeutical options using for example immunotherapy, vaccines, targeted therapy along with the incorporation of biomarkers are ongoing (see paragraph 1.6).

1.6 Molecular abnormalities in HPV positive and negative HNSCC

The heterogeneous nature of HNSCC at the molecular level has hindered both the identification of specific driver mutations and the development of targeted therapeutics. Recent whole-exome sequencing studies have revealed a wide spectrum of genetic aberrations in HNSCC and underscore the molecular diversity of these tumors.[42-44] Figure 1.1 shows the most common abnormalities in HNSCC according to the analysis of The Cancer Genome Atlas (TCGA) Network, including genes in the RTK/RAS/PI3K, cell death, immunity, differentiation and oxidative stress pathways.

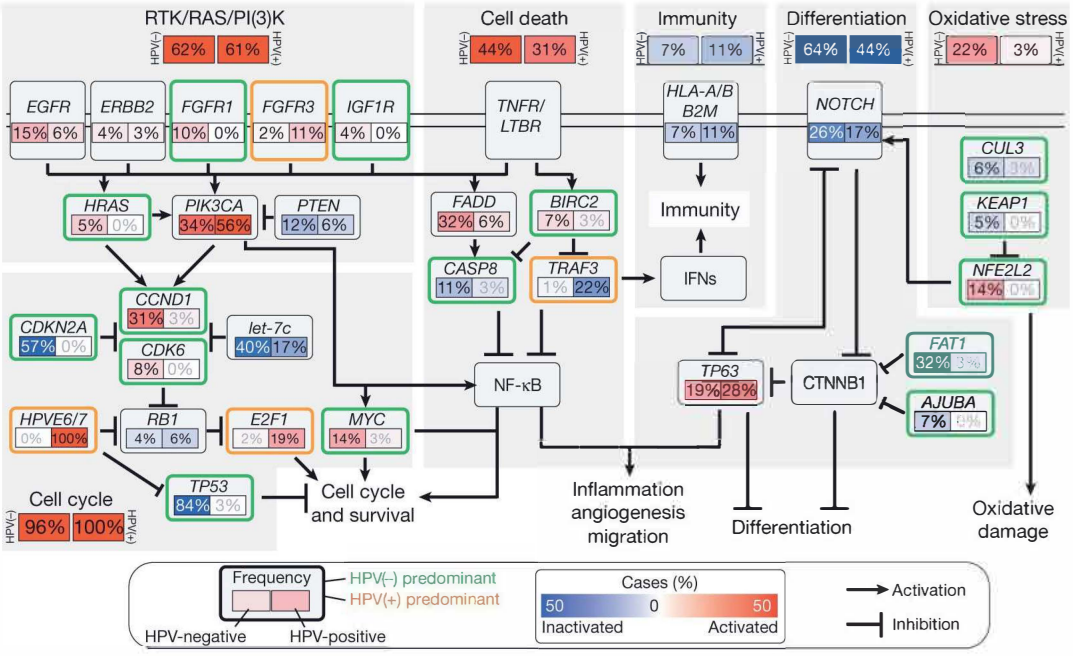

Figure 1.1. Deregulation of signaling pathways and transcription factors. With permission from Dr. D. Hayes; The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature 2015;517:576–82, adapted from figure 5.

In this thesis, a number of inhibitors affecting the cell cycle, survival and cell metabolism are investigated for their efficacy to inhibit HNSCC cell growth and apoptosis.

1.6.1 Cell cycle

The retinoblastoma tumor suppressor protein Rb1 plays a critical role in regulating cellular proliferation. Cyclin D-cyclin-dependent kinase 4/6 (CDK4/6) phosphorylates and inactivates Rb1, leading to release and activation of E2F transcription factors, necessary for G1-S phase cell-cycle progression. The most common abnormalities in HNSCC according to the TCGA data in cell cycle control are mutations in genes TP53 at 84%, CDKN2A at 58% and CCND1 at 31% (mostly amplifications).[44] Loss of CDKN2A (for example, by gene mutation or promotor hypermethylation in combination with loss of heterozygosity, or by homozygous deletion), encoding the p16INK4A protein, or amplification of CCND1 (gene on chromosome 11q13 encoding Cyclin D1, the regulatory subunit of the complex) leads to hyperactivation of the CDK4/6-Cyclin D complex and thereby inactivation of Rb1, which drives cells through the G1-S checkpoint of the cell cycle and contributes to unscheduled DNA replication. These genes are most frequently altered in HPV negative HNSCC and unaffected in HPV positive HNSCC. In the latter tumors the binding of the HPV E6 and E7 proteins to p53 and Rb1 leads to the inactivation of the p53 and p16INK4A-cyclin D-Rb pathway respectively[45,46] (see Figure 1.2A).

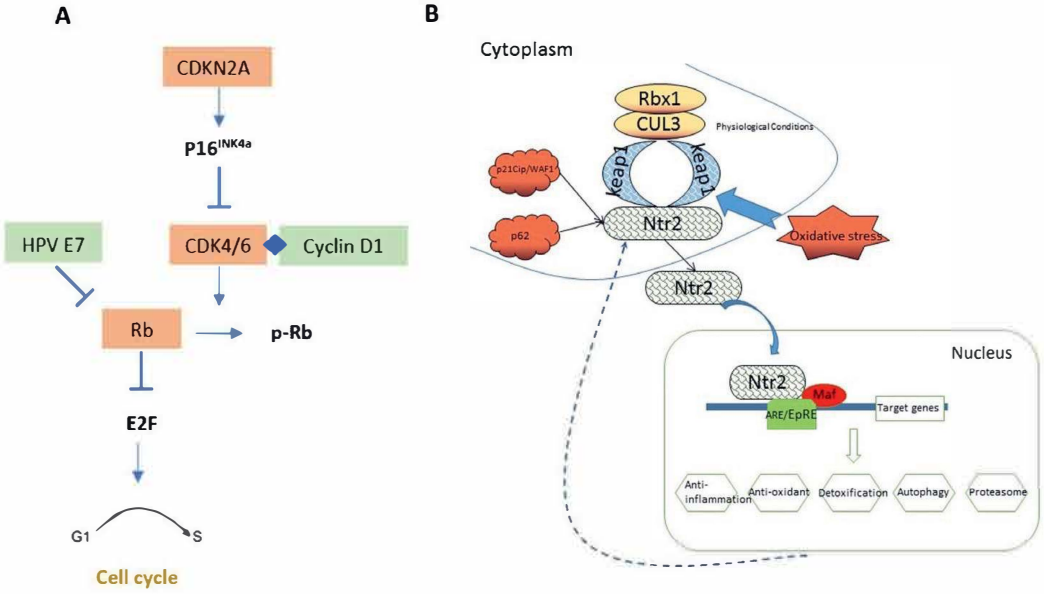

Figure 1.2. (A) The Cdk4/6-Cyclin D pathway (for a description see paragraph 1.6.1) and (B) The KEAP-Nrf2-ARE pathway (for a description see paragraph 1.6.3) (With permission from Wenjun Tu, The Anti- Inflammatory and Anti-Oxidant Mechanisms of the Keap1/Nrf2/ARE, Aging Dis. 2019;10(3):637-51, adapted from figure 3)

1.6.2 Survival

The phosphoinositide 3-kinase (PI3K) pathway affects transcription and translation of multiple targets that are involved in various cellular properties such as survival, proliferation and motility. In a normal cell, activation of PI3K leads to the synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) at the plasma membrane and subsequently to the recruitment of the pleckstrin homology domain-containing proteins phosphoinositide dependent protein kinase-1 (PDK1) and Akt. PDK1 phosphorylates Akt at threonine 308 and activates Akt and other downstream signaling elements, like mammalian target of rapamycin (mTOR) complex 1. Akt regulates transcription factors to allow expression of pro-survival genes and influences many factors involved in apoptosis, either by transcriptional regulation or direct phosphorylation. For example, phosphorylation of Forkhead Box O (FoxO) by Akt inhibits transcriptional functions of FoxO and contributes to cell survival, growth and proliferation. The Cancer Genome Atlas (TCGA) data from HPV positive and negative HNSCC patients have demonstrated that more than 50% of the tumors have activated PI3K signaling and related pathways due to mutations in PIK3CA, loss of PTEN, or activation of receptor tyrosine kinases (RTKs).[27] PIK3CA encodes for the PI3K catalytic subunit alpha and PTEN acts as a tumor suppressor. Molecular alterations in these genes are the most common genetic changes in HPV-driven HNSCC, but they are also recurrently found in HPV negative HNSCC.[42,44,47] Therefore, PI3K signaling seems to constitute a driver for HNSCC, independent of HPV status.

1.6.3 Oxidative stress response pathway

Reactive Oxygen Species (ROS) is a term that denotes a series of products produced during the oxidative metabolism of cells. ROS in cancer cells play a vital role in regulating cell death, DNA repair, metabolic reprogramming and tumor microenvironment. The gene NFE2L2 displays missense mutations in HNSCC and encodes for Nuclear factor erythroid 2-related factor 2 (NRF2) (see Figure 1.2B). ROS activated pathways lead to an activation of NRF2. NRF2 acts as a significant transcription factor regulating the antioxidant response, through inducing expression of genes bearing an antioxidant response element (ARE).[48] Known consequences of NRF2 activation are for example activated PI3K pathway signaling, deficiency in autophagy, impaired DNA damage response, and metabolic reprogramming and chemotherapeutic drug modification resulting in resistance to therapy. Besides mutations in NRF2, also mutations in genes encoding its inhibitors, namely the ubiquitin ligase components kelch-like ECH-associated protein 1 (KEAP1) and cullin 3 (CUL3), are identified in some HNSCCs.[44]

1.7 Aim of this thesis

An HPV vaccine has been available for some time now, which could reduce HPV associated HNSCC in the near future. Therefore, it is important that patients and health care professionals are aware of the human papillomavirus, the association of the virus with cancer and the HPV vaccine in order to increase the HPV vaccination coverage.
Despite improvements in detection and treatment of HNSCC, the mortality rates have hardly decreased over the last decades. In order to improve the prognosis of HNSCC patients, there are new therapeutical strategies necessary. There are different pathways which deregulation plays a role in the development of HPV positive and -negative HNSCC. Knowledge about these pathways may pave the way for the development of new treatments for HNSCC. The most common abnormalities in HNSCC according to the analysis of The Cancer Genome Atlas (TCGA) are involved in the cell cycle, survival and oxidative stress response. In addition, antiviral agents such as Cidofovir could also play a role in the treatment of HPV positive and HPV negative HNSCC, however, the mechanism of action of Cidofovir in the inhibition of HPV positive and negative HNSCC is still unclear. HPV positive patients have a more favorable prognosis, however, the use of HPV as a biomarker for dose de-escalation or changing of treatment modalities has not been proven. How HPV integration affects its host cell and whether HPV integration contributes to the prognosis in HNSCC is still unclear.

The following questions were addressed:

1. What is the knowledge among the general population about oropharyngeal carcinoma, and the association of HPV with oropharyngeal carcinoma?
   In order to give an answer to this question, an online cross-sectional survey was used and sent to a representative reflection of the Dutch population aged 18 and older.

2. What is the knowledge among general practitioners about oropharyngeal carcinoma, and the association of HPV with oropharyngeal carcinoma?
   In order to give an answer to this question, a cross-sectional survey was administered to 900 general practitioners and analysed.

3. Are PI3K/Akt/mTOR inhibitors and/or CDK4/6 inhibitors promising agents in the treatment of HPV positive and –negative HNSCC cell lines?
   For this purpose, the efficacy of different PI3K/mTOR protein inhibitors (alpelisib, buparlisib, gedatolisib) and CDK4/6 inhibitors (ribociclib, palbociclib) were

investigated in HPV positive and -negative HNSCC cell lines by using cell growth, cell death, metabolic and Western blotting assays.

4. Could the antiviral agent Cidofovir play a role in the treatment of HPV positive as well as -negative HNSCC cell lines?
   Therefore, HPV positive and -negative HNSCC cell lines were treated with cidofovir and the efficacy was assessed using cell growth, cell cycle, cell death/ mitotic catastrophe, Western blotting and immunofluorescence assays.

5. Are there human genes which expression is influenced by viral integration in HPV positive OPSCC and do they have clinical relevance?
   To answer this question, a genome wide-screen was performed on fresh-frozen HPV16 positive OPSCC samples containing integrated or episomal virus. The expression of genes identified have been validated on a cohort of HPV positive and -negative OPSCC and correlated with clinical data.

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Chapter 2

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

This chapter was published as:
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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 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 in the United States suggests 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 user, 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

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.

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 2.3 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 factor 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 suggests 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 in time to the physician. 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 (Table 3.3). 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 and our study population (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	> 0	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 ☐ GP ☐ Other (please state)

2. Gender ☐ Female ☐ Male

3. Years since graduation as GP

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

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

   ☐ Poor ☐ Sufficient ☐ Good ☐ Very good

5. 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.

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

7. 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			

8. 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.

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

☐ Yes ☐ No ☐ Don't know

10. 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

11. 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

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

This chapter is submitted as:
Verhees F*, Demers I*, Legemaate I, Jacobs F, Hoeben A, Kremer B, Speel EJM. 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.

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Chapter 5

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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Verhees F, Legemaate D, Demers I, Jacobs R, Haakma WE, Rousch M, Kremer B, Speel EJ. The Antiviral Agent Cidofovir Induces DNA Damage and Mitotic Catastrophe in HPV-Positive and -Negative Head and Neck Squamous Cell Carcinomas In Vitro. Cancers (Basel). 2019 Jun 30;11(7):919.

Abstract

Background

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.

Methods

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).

Results

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 amount of mitoses but an increase in aberrant mitoses suggesting mitotic catastrophe.

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 GP51/61.[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 immunofluorescence (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 S5.1) 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 S5.1).

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 electrotransferred 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

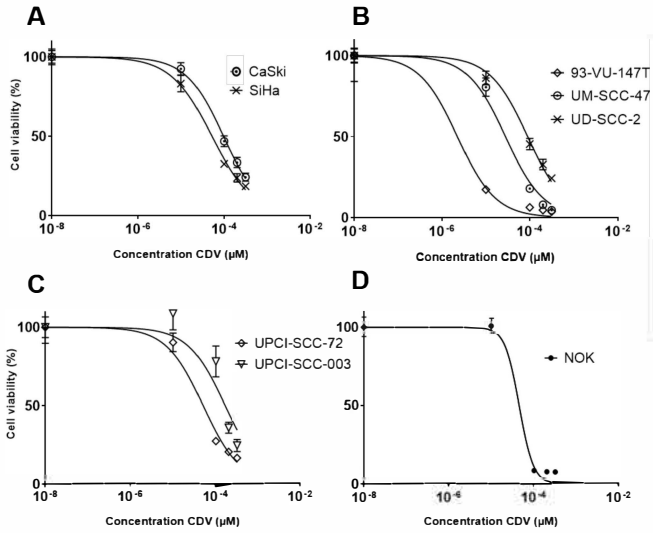
GraphPad Prism (version 6, San Diego, CA, USA) was used to conduct all statistical analyses. All results were expressed as the mean ± standard error of the mean. Independent experiments were analyzed by an unpaired Student's t-test. Levels of $p<0.05$ were considered to be of statistical significance.

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 5.1). 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 5.1 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 S5.1).

All four cell lines were treated for 3 and 6 days with CDV and processed for γ-H2AX immunofluorescence. Figure 5.2A 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 5.2B). The increased expression of phospho-H2AX (p-H2AX) in CDV treated cells was also seen in western blot analyses (Figure 5.2C). 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).

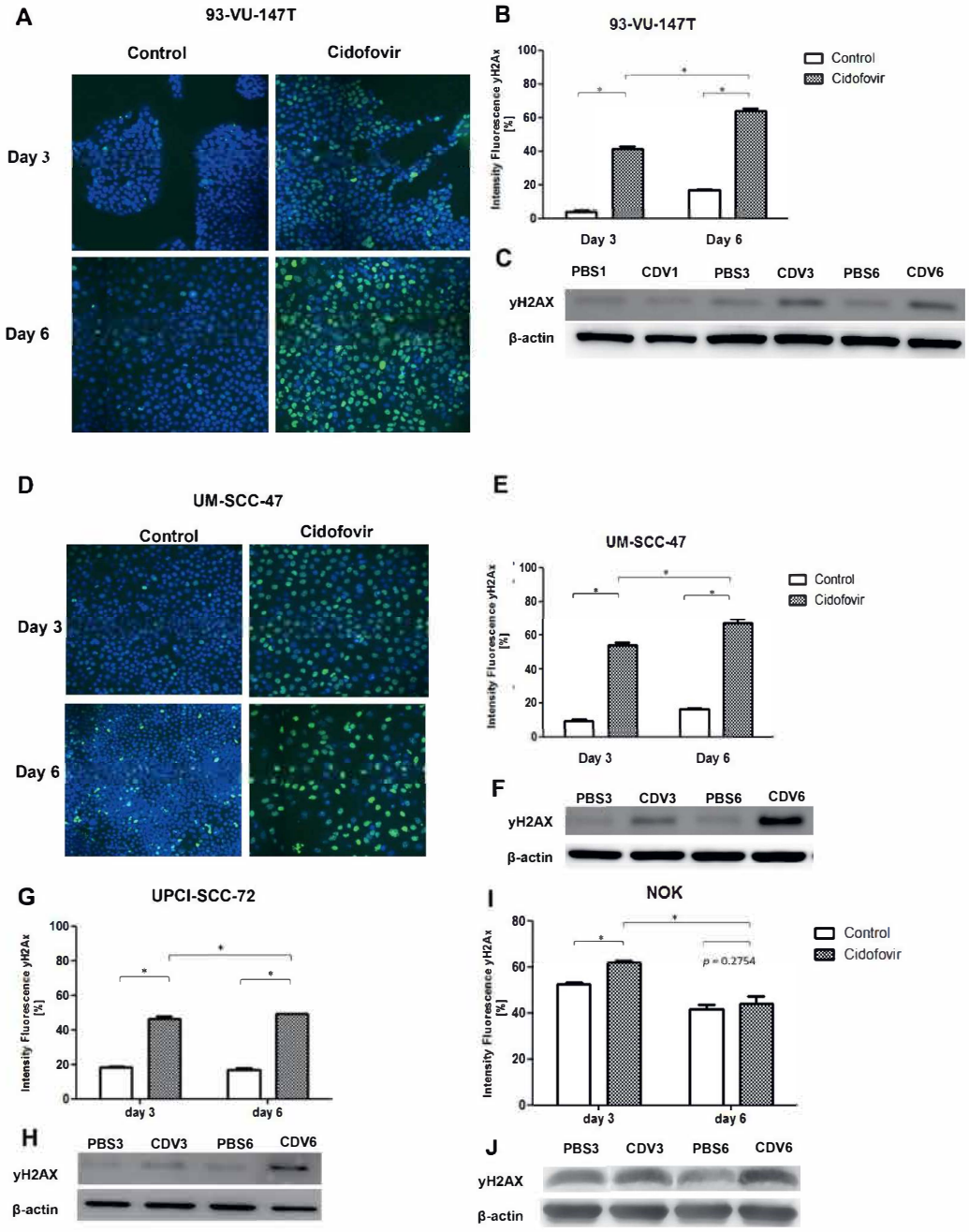


Figure 5.2 DNA damage induced by CDV as detected by γ-H2AX analysis. Cells were treated with CDV or PBS (control) and after 3 and 6 days immunostaining of γ-H2AX was performed. (A) DNA-damage is accumulated in the treated 93-VU-147T cells. Nuclei are stained with Hoechst in blue, DSBs are shown by γ-H2AX 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 5.3A). 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 5.3B). 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 5.3C). 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%).

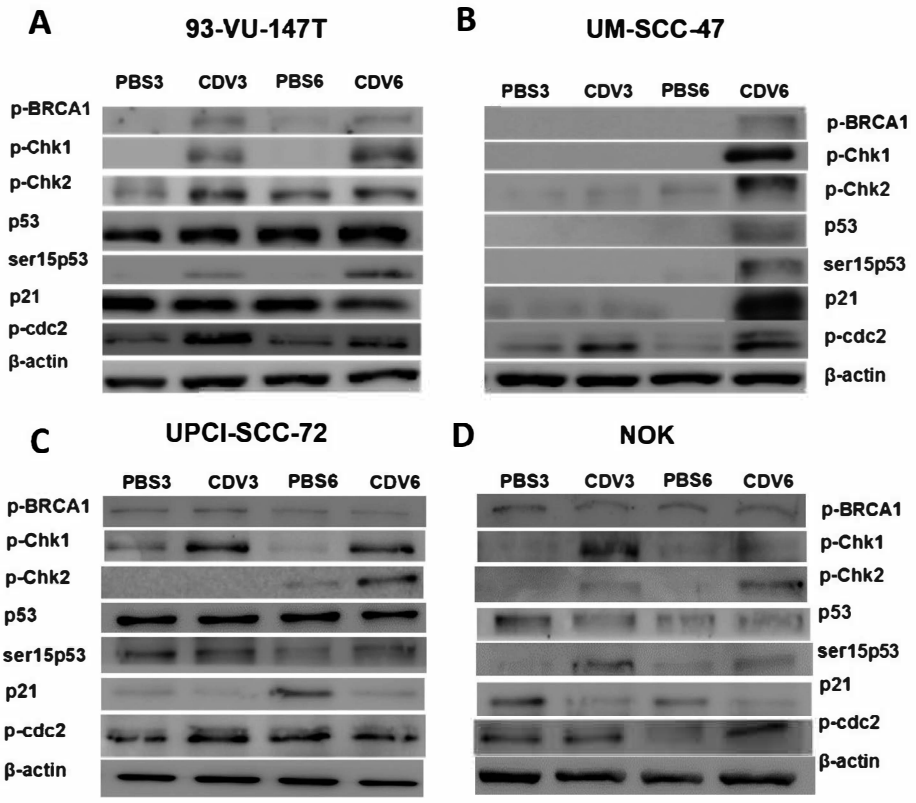


Figure 5.3 Expression levels of proteins involved in the DNA damage response pathway by western blot analysis of whole protein extracts. The cells were treated for 3 and 6 days with the IC_{50} value of CDV or control (PBS). β-actin was used as loading control. For the cell lines (A) 93-VU-147T and (B) 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 5.4).

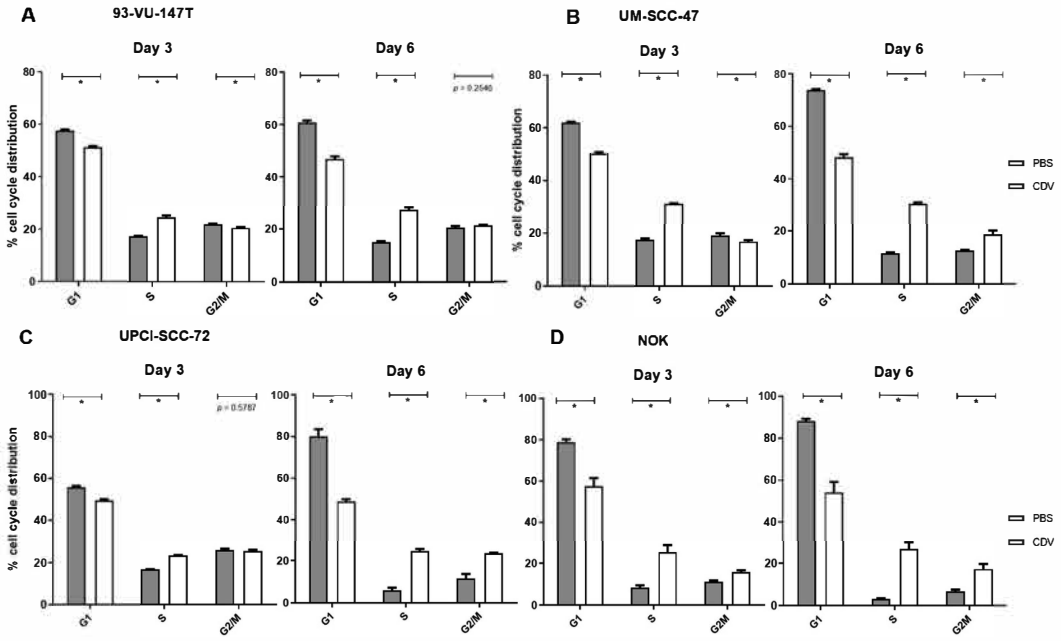


Figure 5.4 Cell cycle distribution of the HNSCC cell lines and NOK treated for 3 and 6 days with CDV or not treated (PBS). (A) 93-VU-147T, (B) UM-SCC-47, (C) UPCI-SCC-72, (D) NOK. Statistical significance was indicated as follows: $p < 0.05$ (*). The experiments were performed in triplicate.

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 5.5). 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 5.6).

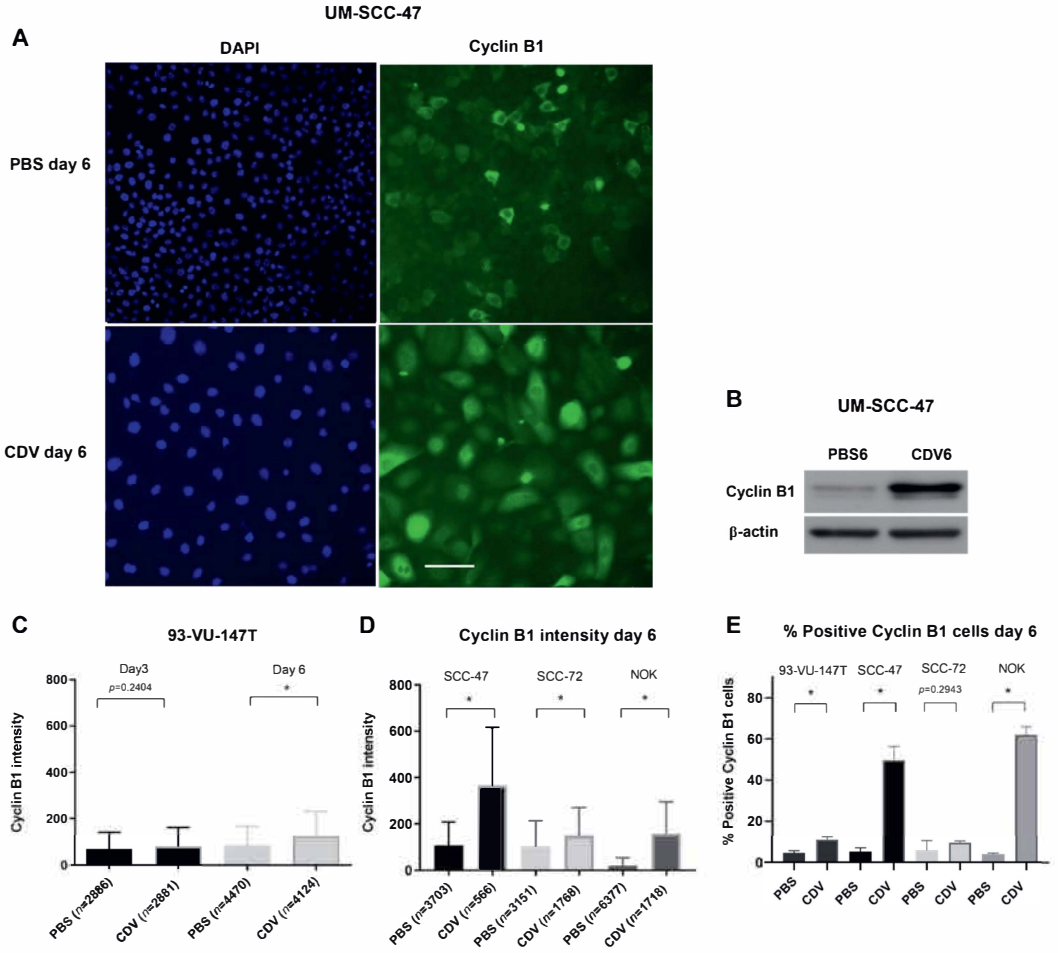


Figure 5.5 Upregulation of cyclin B1 expression in the nucleus after treatment of cell lines with CDV. The cells were treated for 3 and 6 days with the IC_{50} value of CDV followed by cyclin B1 immunofluorescence staining. Nuclei were considered positive if the intensity was higher than the average 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.

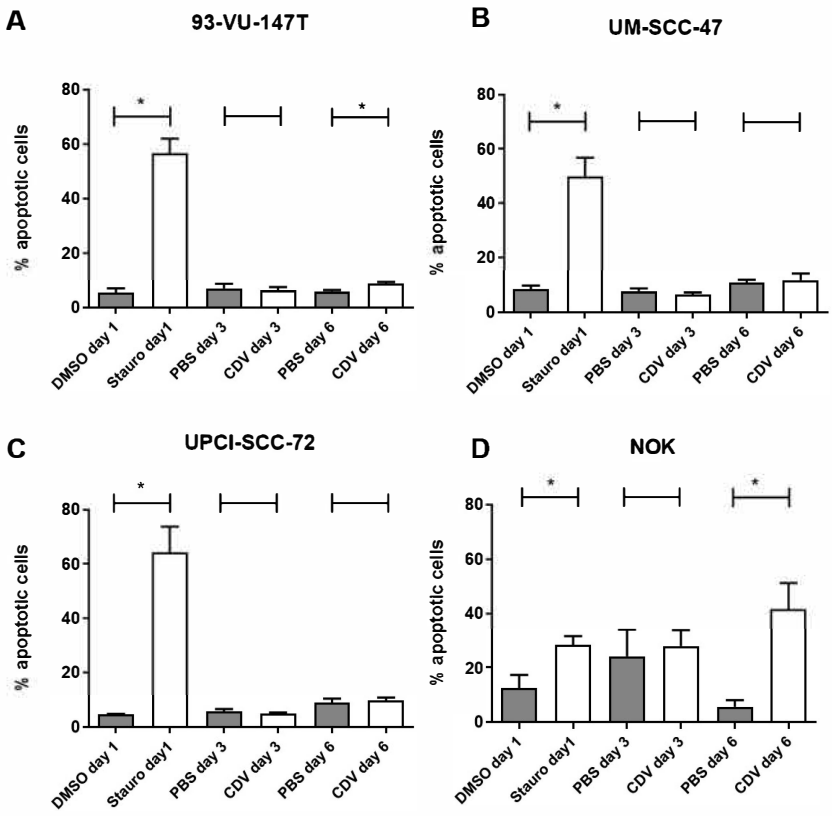


Figure 5.6 Effect of CDV treatment on induction of apoptosis. Cells were either treated for 1 day with 1µM Staurosporine, a known inducer of apoptosis or for 3 and 6 days with CDV, followed by analysis of Annexin V staining. Results are shown for (A) 93-VU-147T, (B) UM-SCC-47, (C) UPCI-SCC-72, and (D) 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 were an increase in cell nuclei size after CDV treatment in comparison with the control cells (Figure S5.2). CDV treated cells showed a decrease in number of mitotic figures and an increase in cells in mitotic catastrophe (Figure 5.7). 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 5.7I shows that more mitotic catastrophes were observed with increasing sensitivity for CDV.

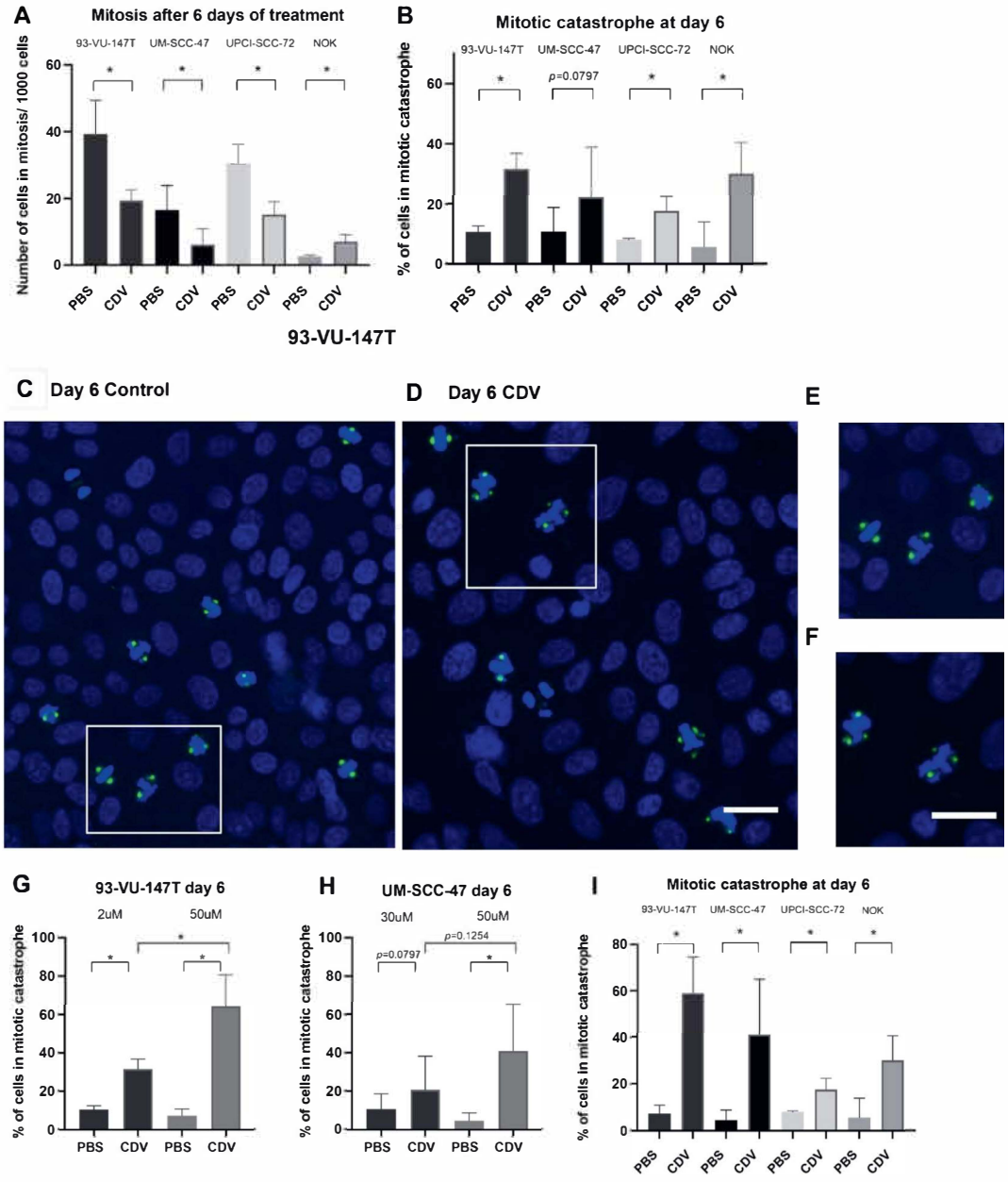


Figure 5.7 Induction of mitosis and mitotic catastrophe after treatment with CDV. The cells were treated with CDV or PBS for 3 and 6 days after which immunostaining of phospho-Aurora Kinase was performed. The cells were treated with an equal toxicity (IC_{50}) and with the same CDV concentration (50 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.

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 materials

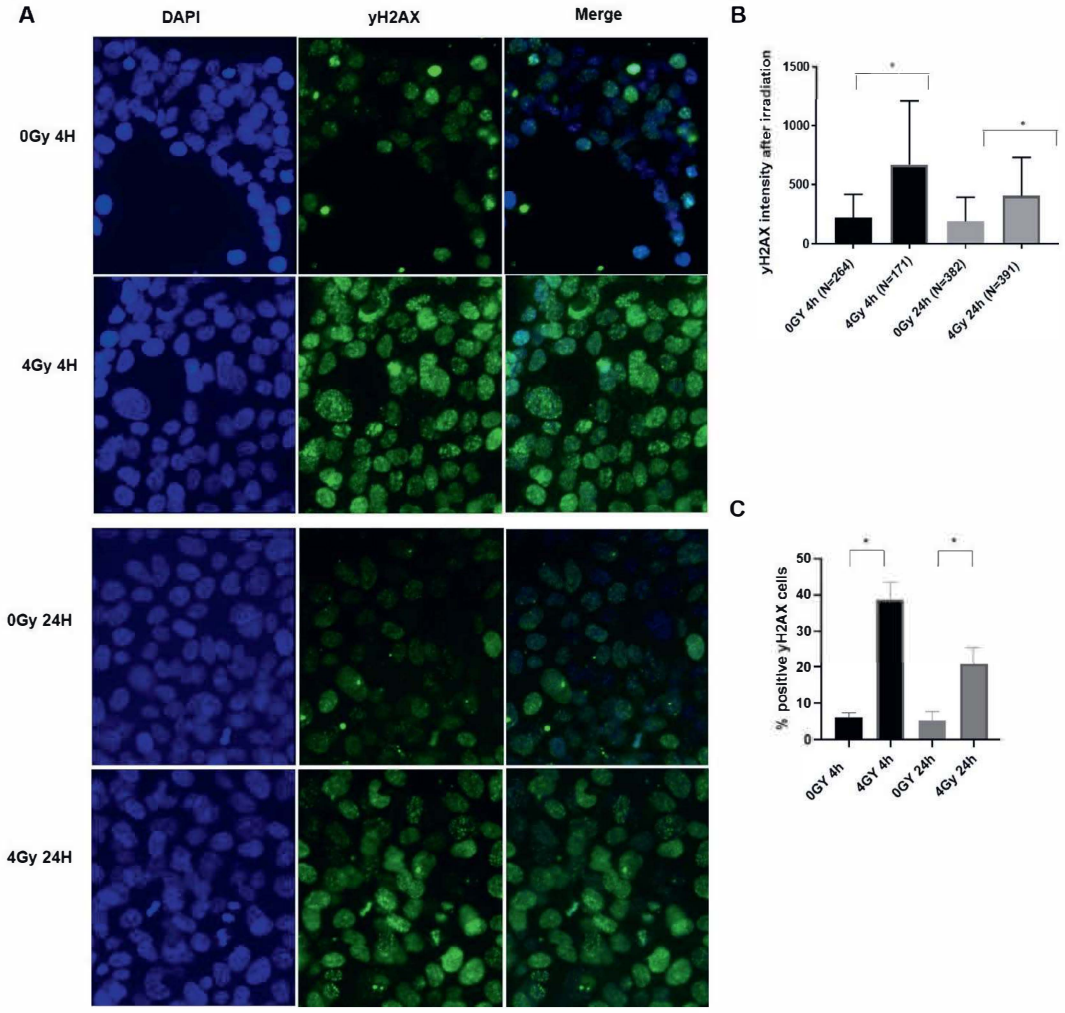

Figure S5.1 (**A**) The occurrence of DNA-damage in 93-VU-147T treated with 4 Gray irradiation in vitro (magnification ×200). After irradiation, the cells were cultured for 4 and 24 hours and analyzed for immunofluorescence with γ-H2AX. Nuclei are stained with DAPI in blue. DNA double strand breaks (DSBs) 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: $p<0.05$ (*).

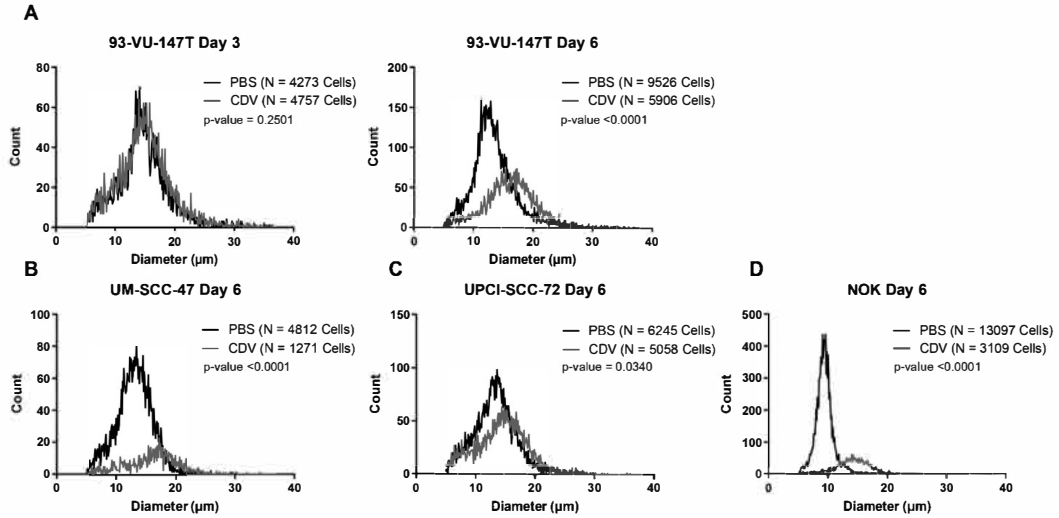

Figure S5.2 Effect of CDV treatment on the cell nucleus diameter. The cells were treated for 3 and 6 days with the IC50 value of CDV followed by immunofluorescence staining of Cyclin B1 or phospho-Aurora Kinase. After 6 days there is a significant increase in cell nucleus diameter in the different cell 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 S5.1 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 6

Upregulation of AKR1C1 and AKR1C3 expression in OPSCC with integrated HPV16 and HPV-negative tumors is an indicator of poor prognosis

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Abstract

Background

Different studies have shown that HPV16-positive OPSCC can be subdivided based on integration status (integrated, episomal and mixed forms). Because we showed that integration neither affects the levels of viral genes, nor those of virally disrupted human genes, a genome-wide screen was performed to identify human genes which expression is influenced by viral integration and have clinical relevance.

Methods

Thirty three fresh-frozen HPV-16 positive OPSCC samples with known integration status were analyzed by mRNA expression profiling. Among the genes of interest, Aldo-keto-reductases 1C1 and 1C3 (AKR1C1, AKR1C3) were upregulated in tumors with viral integration. Additionally, 141 OPSCC, including 48 HPV-positive cases, were used to validate protein expression by immunohistochemistry. Results were correlated with clinical and histopathological data.

Results

Non-hierarchical clustering resulted in two main groups differing in mRNA expression patterns, which interestingly corresponded with viral integration status. In OPSCC with integrated viral DNA, often metabolic pathways were deregulated with frequent upregulation of AKR1C1 and AKR1C3 transcripts. Survival analysis of 141 additionally immunostained OPSCC showed unfavorable survival rates for tumors with upregulation of AKR1C1 or AKR1C3 (both $p<0.0001$), both in HPV-positive ($p\leq0.001$) and -negative ($p\leq0.017$) tumors.

Conclusion

OPSCC with integrated HPV16 show upregulation of AKR1C1 and AKR1C3 expression, which strongly correlates with poor survival rates. Also in HPV-negative tumors, upregulation of these proteins correlates with unfavorable outcome. Deregulated AKR1C expression has also been observed in other tumors, making these genes promising candidates to indicate prognosis. In addition, the availability of inhibitors of these gene products may be utilized for drug treatment.

Introduction

Worldwide, head and neck squamous cell carcinoma (HNSCC) is the sixth most common and one of the most lethal types of cancers. Patients frequently develop unfavorable distant metastases and inoperable local and regional recurrences.[1] Even though improvements in detection and clinical treatment (including surgical intervention, radiation and chemotherapy) have been achieved, prognosis of this malignancy with only 40-50% of patients surviving the next 5 years after initial diagnosis is still unfavorable. The most prominent risk factors for the development of HNSCC are excessive tobacco and alcohol use, as well as high-risk human papillomavirus (HPV) infections.

Most frequently, HPV16 has been found to be associated with oropharyngeal squamous cell carcinoma (OPSCC).[2,3] This subgroup of carcinomas shows clinicopathological and molecular characteristics that differ from alcohol- and tobacco-induced carcinomas.[4] A question that still remains to be elucidated is if there is a biological consequence of viral integration. So far, there is little evidence that viral integration may have an impact on prognosis[5-10], furthermore, in previous work of our group no evidence was found that HPV integration significantly affects viral gene expression or the expression of disrupted human genes as compared to tumors with episomal virus.[3,11-13] Thus, it remains to be identified by which mechanisms HPV integration affects its host cell and if they are of clinical relevance.

Our aim was to perform a genome-wide screen to identify human genes whose expression is influenced by integration of HPV16. For this purpose, we compared HPV16-positive OPSCC harboring extrachromosomal or integrated virus DNA using mRNA microarray expression profiling.

We identified a unique signature of differentially expressed human mRNAs in relation to viral physical state. Selected candidate genes comprised the AKR1C1 and AKR1C3 genes which expression was independently confirmed by RT-qPCR as well as immunohistochemistry using both part of the screening cohort and a new cohort of 141 OPSCC.

Methods

Subjects and material

Cohort 1: Fresh frozen clinical OPSCC samples positive for HPV16 from 33 patients treated at the Departments of Otorhinolaryngology and Head and Neck Surgery of the University Hospitals of Cologne and Maastricht between 1994 and 2009 were collected

from the archives of the Departments of Pathology of both hospitals (Supplementary Table S6.1). This cohort was selected out of a previously published group of 75 patients based on the following inclusion criteria: ≥70% tumor cells, sufficient high-quality DNA and RNA (RQI value ≥7; BioRad Experion System, BioRad Laboratories Munich, Germany) and positive HPV-PCR and p16INK4A Immunostaining 3. These 33 patients were also analyzed by APOT and / or DIPS PCR for viral integration status, which revealed 9 (27.3%) cases with only integration, 4 (12.1%) cases with both integrated and episomal virus and 20 (60.6%) cases with episomal virus.[3]

Cohort 2: Formalin-fixed, paraffin embedded (FFPE) tissue samples from 141 patients with primary OPSCC (2000-2011) containing both tumorous and adjacent tumor free tissue were available from the Departments of Otorhinolaryngology, Head and Neck Surgery, and Pathology, University of Cologne. HPV-status (HPV-PCR and p16INK4A Immunostaining) was available for all 141 patients. 93 patients (66.0%) were HPV-negative, 48 (34.0%) HPV-positive (Table 6.1).

Ethics statement

Patient material was used according to the code for proper secondary use of human tissue. The ethics committee of the Medical Faculty of the University of Cologne approved this study (approved protocol no. 11-346). Written, informed consent had been obtained from all patients.

Identification of HPV16 integration status

To identify viral integration status in Cohort 1, both a mRNA based assay (Amplification of Papillomavirus Oncogene Transcripts PCR; APOT-PCR) and a DNA based assay (Detection of Integrated Papillomavirus Sequences-PCR; DIPS-PCR) were performed as described previously.[3]

Table 6.1 Summary of clinicopathological features of patients analyzed in cohort II of our study.

			HPV-Status					AKR1C1 Staining					AKR1C3 Staining				
	Total [1]		HPV+		HPV-			AKR1C1 + [2]		AKR1C1 - [2]			AKR1C3 + [2]		AKR1C3 - [2]		
Clinicopathological feature	**n**	**%**	**n**	**%**	**n**	**%**	**χ^2**	**n**	**%**	**n**	**%**	**χ^2**	**n**	**%**	**n**	**%**	**χ^2**
Mean age (years)	141		56.7		60.1		0.130	55.6		58.4		0.165	55.1		58.3		0.122
Gender																	
Male	103	73.0	36	25.5	67	47.5		42	29.8	61	43.3		35	24.8	68	48.2	
Female	38	27.0	12	8.5	26	18.4	0.674	15	10.6	23	16.3	0.889	13	9.2	25	17.7	0.980
T classification																	
pT1 and pT2	79	56.0	32	23.5	45	33.1		28	19.9	51	36.1		23	16.3	56	39.7	
pT3 and pT4	62	44.0	16	11.8	43	31.6	**0.040**	29	20.6	33	23.4	0.220	25	17.7	37	26.2	0.204
N classification																	
pN0	28	19.9	8	5.8	19	13.8		11	7.8	17	12.1		9	6.4	19	13.5	
pN1-2 [3]	113	80.1	40	29.0	71	51.4	0.654	47	33.3	66	46.8	0.668	41	29.1	72	51.1	0.654
M classification																	
pM0	131	92.9	46	34.1	79	58.5		52	36.9	79	56.0		45	31.9	86	61.0	
pM1	10	7.1	2	1.5	8	5.9	0.494	6	4.3	4	2.8	0.318	4	2.8	6	4.3	0.739
AJCC classification																	
I	26	19.1	18	12.8	8	5.7		4	2.8	27	19.1		4	2.8	23	16.3	
II	20	14.9	16	11.3	4	2.8		8	5.7	13	9.2		7	5.0	14	9.9	
III	27	19.1	9	6.4	18	12.7		14	9.9	13	9.2		14	9.9	13	9.2	
IV	55	46.8	5	3.5	51	43.2	**<0.0001**	31	22.0	35	24.8	**0.024**	24	17.0	42	29.8	0.063
Grading																	
1	1	0.7	0	0	1	0.7		1	0.7	0	0		0	0	1	0.7	
2	86	60.1	27	20.1	54	40.3		38	26.9	48	34.0		34	24.1	52	36.9	
3	54	38.3	20	14.9	32	23.9	0.634	20	14.2	34	24.1	0.330	15	10.6	39	27.7	0.202
Surgery																	
Yes	110	78.1	39	30.0	62	47.7		42	29.8	68	48.2		36	25.5	74	52.5	
No	31	21.9	5	3.8	24	18.5	**0.044**	16	11.3	15	10.6	0.173	13	9.2	18	12.8	0.325
Radiotherapy																	
Yes	106	76.3	31	24.6	69	54.8		40	28.8	66	47.5		33	23.7	73	52.5	
No	33	23.7	9	7.1	17	313.5	0.814	17	12.2	16	11.5	0.160	15	10.8	18	12.9	0.131

Table 6.1 (continued)

			HPV-Status					AKR1C1 Staining					AKR1C3 Staining				
	Total [1]		HPV+		HPV-			AKR1C1 + [2]		AKR1C1 - [2]			AKR1C3 + [2]		AKR1C3 - [2]		
Clinicopathological feature	**n**	**%**	**n**	**%**	**n**	**%**	**χ^2**	**n**	**%**	**n**	**%**	**χ^2**	**n**	**%**	**n**	**%**	**χ^2**
Chemotherapy																	
Yes	71	51.1	23	18.9	42	34.4		27	19.4	44	31.7		20	14.4	51	36.7	
No	68	48.9	15	12.3	42	34.4	0.330	30	21.6	38	27.3	0.466	28	20.1	40	28.8	0.107
Relapse																	
Yes	58	41.1	14	9.9	44	31.2		37	26.2	21	14.9		34	24.1	24	17.0	
No	83	58.9	34	24.1	49	34.8	**0.039**	21	14.9	62	44.0	**<0.0001**	15	10.7	68	48.2	**<0.0001**
Death																	
Yes	42	36.9	11	7.8	41	29.1		32	22.7	10	7.1		31	22.0	11	7.8	
No	89	63.1	37	26.2	52	36.9	**0.024**	17	12.1	82	58.2	**<0.0001**	16	11.3	83	58.9	**<0.0001**
HPV-status																	
Negative	93	66.0						30	21.3	54	38.3		35	24.8	62	44.0	
Positive	48	34.0						14	9.9	30	21.3	0.161	13	9.2	31	22.0	0.448
Smoking																	
Yes	99	86.1	23	21.3	69	63.9		40	34.8	59	51.3		34	29.6	65	56.5	
No	16	13.9	14	13.0	2	1.9	**<0.0001**	4	9.1	12	10.4	0.280	2	1.7	14	12.2	0.093
Alcohol																	
Yes	92	80.0	21	19.4	64	59.3		39	33.9	53	46.1		33	28.7	59	51.3	
No	23	20.0	16	14.8	7	6.5	**<0.0001**	5	4.3	18	15.7	0.093	3	2.6	20	17.4	**0.044**
Localization																	
Base of tongue	20	14.2	11	7.8	9	6.4		7	4.9	13	9.2		5	3.5	15	10.6	
Tonsil	90	63.8	34	24.1	56	39.7		40	28.4	50	35.5		35	24.8	55	39.0	
Remaining Oropharynx	31	22.0	4	2.8	27	19.1	**0.006**	10	7.1	21	14.9	0.440	10	7.1	21	14.9	0.360

Summary of clinicopathological features of patients analyzed in our study. n = Number of patients. Staging was performed according to AJCC/UICC 8th Edition in Oropharyngeal Squamous Cell Carcinoma.

[1] Total number corresponds to the maximal number of patients analyzed. In the HPV-positive group, data from 129 patients were available; [2] Relative staining compared to normal epithelium. + means higher expression in tumor cells compared to normal epithelium, - means less or equal staining in tumor cells compared to normal epithelium; [3] More than one site of the Oropharynx harbored tumor.

χ^2: Chi-Square test for significance. For mean age, ANOVA is used to measure significance. Significant values are highlighted in bold.

mRNA expression profiling

Total RNA was collected from a subset of 33 samples of appropriate mRNA quality, selected from 75 patients reported in a previous study (Cohort 1[3]). Samples were analyzed using Agilent Whole Human Genome 4 x 44K Microarrays, which represent more than 41,000 unique human transcripts. Labelling and hybridizations were performed according to the manufacturer's instructions (Agilent Technologies). Hybridized arrays were scanned using an Axon GenePix 4000B or 4200A scanner. Microarray analysis was performed using GenePix Pro 6.0.1.25. For normalization processing, the median array intensity was calculated based on the background-subtracted intensity value for all spots excluding control type spots on the array. The background-subtracted intensity value of each spot was then divided by the median array intensity of each microarray. In the 33 tumor samples, 9 samples harbored exclusively integrated HPV16 DNA and the remaining 24 samples harbored episomal viral DNA irrespective of further integrated viral DNA (20 samples episomal viral DNA, 4 samples episomal and integrated viral DNA). Bioinformatic analysis was performed using Partek genomic suite (Partek, St. Louis, Missouri) using unsupervised clustering. Samples with a differential expression (2.0-fold cut-off filter) and $p \leq 0.05$ (student's t-test) were filtered for confidence followed by Benjamini-Hochberg false discovery rate (FDR) correction (FDR ≤0.1) and selected for further analysis (Supplementary Tables S6.2 and S6.3). Interaction networks were calculated using Cytoscape including the plugin ClueGo for analysis of Gene-Ontology (GO) and pathways.[14] Primary mRNA array data have been made publicly available at EMBL-EBI (Accession No. E-MTAB-6350) for use in subsequent analysis.

RT-qPCR expression analysis

500 ng of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad Laboratories Munich, Germany). qPCR reactions were performed using 1 µl of the 20 µl RT product with iTaq Universal SYBR Green Supermix (BioRad). Amplification was performed for 40 cycles with initial denaturation at 95 °C for 5 min, followed by cycles with 15 sec at 95°C, 30 sec at primer specific temperatures for annealing and 40 sec at 72 °C followed by melting curve analysis. Specific primers are summarized in Supplementary Table S6.4. The detection of the housekeeping gene Hypoxanthine Phosphoribosyltransferase (HPRT) was used for normalization of mRNA levels. Human mRNAs from pancreatic islands, complete pancreas, lung, liver and adrenal gland served as controls (Supplementary Figures S6.1J and S6.2J).

Immunohistochemistry

Immunohistochemical protein staining on 4 µm thick FFPE tissue sections was carried out as described earlier.[15] Briefly, sections were deparaffinized and subsequently pretreated with 3% H2O2 in methanol for 10 min to quench endogenous peroxidase activity. Antigen retrieval was carried out by heating at 70°C in 0.01 M citrate buffer (pH 6.0) over night. AKR1C1 was detected by mouse monoclonal antibodies (Abcam, clone AT6D10, Cambridge, GB; 1:500 in PBS) and AKR1C3 was detected by mouse monoclonal antibody (Sigma Aldrich, clone NP6.G6.A6, Taufkirchen, Germany, 1:500 in PBS). 3-Nitrotyrosine was detected by mouse monoclonal antibody (Santa Cruz Biotechnol., clone 39B6, Heidelberg, Germany, 1:50 in PBS) and NRF2 by a rabbit polyclonal antibody (Sigma Aldrich, HPA002990, 1:200 in PBS). After incubation with a biotinylated secondary antibody, immunohistochemical detection was performed by an avidin–biotinylated peroxidase complex (ABC) procedure (Vectastain-Elite-ABC kit; Vector, Burlingame, USA). Peroxidase activity was detected using DAB substrate kit (Vector) according to the manufacturers protocol. Sections were counterstained with hematoxylin and mounted in Roti Histokit (Carl Roth, Karlsruhe, Germany). Tonsillar epithelium from routine tonsillectomy, pancreas, liver and placenta served as controls (Supplementary Figures S6.1A-C and S6.2A-C). Antibody specificity was validated by control stainings without primary antibody (Supplementary Figures S6.1 and S6.2D-F) and IgG isotype control antibodies (data not shown). Analysis was carried out by four independent observers (CUH, LP, JK and SFP), and in case of interobserver variations, consensus was reached by combined examination of the slides. The staining intensity of the tumor was evaluated in relation to the staining intensity in the adjacent squamous epithelium.

Statistics

Epidemiological data combined with HPV-status and AKR1C1 and AKR1C3 immunohistochemical staining were analyzed using cross-tabulations, χ^2 test and Fisher's exact probability test with the software SPSS 21.0. Overall survival and disease-free survival rates were estimated using the Kaplan–Meier algorithm for incomplete observations. The overall survival time was measured from the date of diagnosis to the last date when the patient was known to be alive (censored) or date of death for any reason (uncensored). The disease-free survival rate was defined as the period of time beginning on the date of diagnosis to the day of the last follow-up examination in which the patient was disease-free (censored), or to the date of local recurrence of the disease or occurrence of regional or distant metastases (uncensored). The log-rank test was used to perform the univariate analysis of the various variables. A significance level of

p≤0.05 was considered statistically significant in 2-sided tests. Statistical analyses of microarray and RT-qPCR data was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla California, USA) using student's t-test and ANOVA.

Results

mRNA expression profiling in relation to integration status

mRNA expression profiling was performed on 33 HPV16-positive OPSCC with known viral integration status (cohort 1, Supplementary Table S6.1). Unsupervised clustering of mRNA expression data separated the samples into two major clusters (Figure 6.1A). When we annotated the viral integration data to these clustered expression results, one cluster matched almost entirely with tumors harboring exclusively host genome integrated HPV16 DNA (tumors with integrated virus), and the other one with tumors containing extrachromosomal HPV16 DNA (tumors with episomal virus). Three of the four tumors with mixed forms of integrated and episomal virus also clustered within the group with solely episomal viral DNA.

Identification of deregulated genes and pathways

Comparison of mRNA expression profiling of tumor samples with integrated versus episomal virus revealed 470 upregulated and 13 downregulated annotated human mRNAs (≥2-fold deregulation, p≤0.05, FDR≤0.1) (Supplementary Table S6.2 and S6.3).
By using the Cytoscape software and ClueGo plugin we identified 24 affected cellular pathways with deregulated mRNAs, including epidermal development and differentiation, hormone regulation and processing, oxidative stress response and metabolic processes of ketones, organic acids and (mono)carboxylic acids (Figure 6.1B). Interestingly, four human mRNAs were found to be significantly deregulated in multiple affected pathways (Figure 6.1B), i.e. AKR1C1 (upregulated 7.5x, p=0.009 in OPSCC with integrated viral DNA), AKR1C3 (upregulated 7.0x, p=0.023), BCL2 (downregulated 3.0x, p=0.019) and BCL2L10 (upregulated 3.4x, p=0.005). In contrast, the expression of a number of targets known to be involved in HPV-induced carcinogenesis was not significantly correlated with integration status, such as BCLX, CDKN2A, E2F1, EGFR, PIK3CA, RB1, TP53 and TP63.

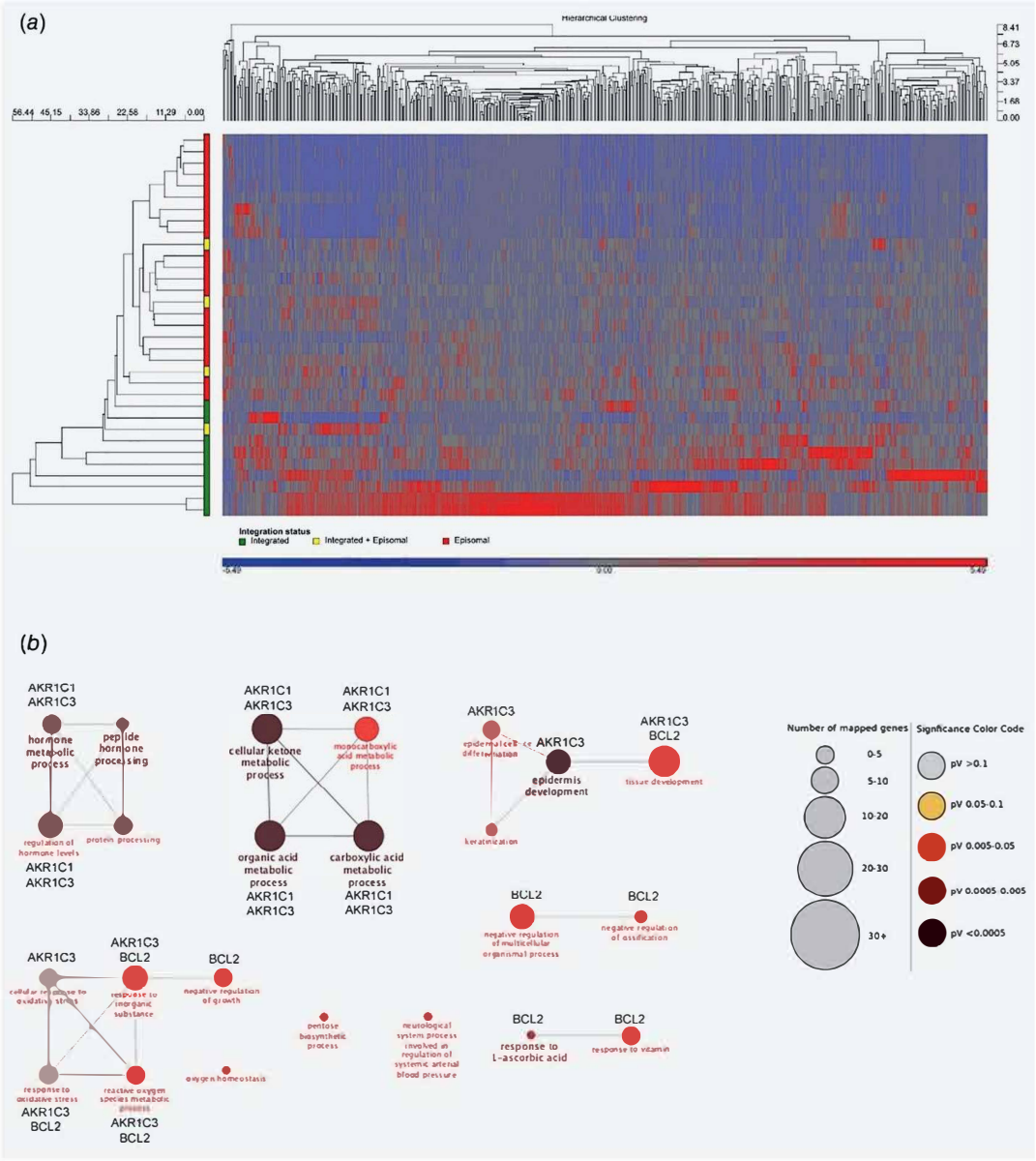

Figure 6.1 (A) Unsupervised gene set cluster analysis of 33 HPV16-positive OPSCC samples with known viral integration status. Hierarchical clustering of differentially regulated genes (fold change ≤2.0 and p-value ≤0.05, FDR≤0.1) allowed to separate tumor samples with integrated (I, green), episomal (E, red) and mixed forms (E + I, yellow). Y-axis represents patient samples, x-axis represents list of probe sets.

(B) Gene-ontology (GO) pathway data analysis. Overview of differentially regulated pathways according to their classification in the GO database. OPSCC with exclusively integrated HPV16 DNA (I) were compared with samples harboring extrachromosomal viral DNA (E and E + I). Gene products of special interest are indicated with pathways they are contributing to.

Confirmation of AKR1C1, AKR1C3, BCL2 and BCL2L10 mRNA expression by RT-qPCR

To confirm the expression level of the four genes identified by GO-term clustering, RT-qPCR was performed on 27 cases out of cohort 1. Similar to mRNA expression profiling, also the RT-qPCR results demonstrated upregulation of AKR1C1, AKR1C3 and BCL2L10 and downregulation of BCL2 in the OPSCC with integrated virus (Supplementary Figure S6.3A-H).

Upregulation of E6*I transcripts in OPSCC with integrated HPV16

Because studies have shown that metabolic pathways including oxidative stress response may be activated by the virus derived splice variant E6*I 16, we analyzed the mRNA isolated from Cohort I tumors for E6*I expression by RT-qPCR (Supplementary Figure S6.3I). E6*I expression showed to be significantly upregulated in OPSCC with exclusively integrated viral genomes (p=0.001). E6*I expression levels in tumors with mixed forms of integrated and episomal virus (n=3) were similar to those detected in tumors with exclusively episomal virus.

Immunohistochemical detection of AKR1C1 and AKR1C3

To further evaluate the microarray findings, we focused on examining the expression of AKR1C1 and AKR1C3 proteins, because these enzymes have been reported as potential targets for therapy and are upregulated in epithelial tissue of smokers and chemoresistant tumors.[17] First, we analyzed the expression in a number of normal human control tissues including liver, pancreas, placenta and tonsils (see Supplementary Figures S6.1 and S6.2 also for some corresponding mRNA expression analysis). For both proteins, high expression levels were detected in liver and exocrine pancreas, pancreatic islands showed both cells with high expression and no or very low expression, and no expression was detected in placenta, which is in agreement with stainings reported in human protein atlas.[18] Both in mRNA and protein expression analysis, AKR1C1 appears to be higher expressed than AKR1C3. In normal tonsils (n=6), lymphocytes were negative and squamous epithelial keratinocytes showed no or weak predominantly nuclear staining, whereas muscle cells and endothelial cells showed strong staining (Supplementary Figures S6.1 and S6.2A-C). Control stainings without primary antibodies and IgG isotype controls were negative in all tissues (Supplementary Figures S6.1 and S6.2D-F and data not shown).

Subsequently, we analyzed 24 OPSCC of cohort I with sufficient FFPE tissue available, namely 16 cases with episomal virus and 8 with integration and detected a similar

variance in protein expression as was found for mRNA by microarray and RT-qPCR analysis when comparing integration status (Supplementary Figure S6.4). Because we noticed some squamous epithelia with positive staining, we also decided to evaluate staining of both tumors and the adjacent epithelia and to relate the staining intensities to each other in further analysis. In these 24 cases, the resulting protein intensity ratios showed a similar pattern as the RT-qPCR results, i.e. lower expression in OPSCC with episomal HPV16 (Supplementary Figure S6.4B and S6.4C).

To evaluate our findings in a separate large cohort (cohort 2), we immunostained 141 OPSCC and their adjacent epithelia including 48 HPV-positive tumors for AKR1C1 and AKR1C3 (Figure 6.2). For AKR1C1, 59 out of 141 OPSCC (41.8%) showed stronger staining in the tumor than in the adjacent epithelium (AKR1C1+). The remaining 82 OPSCC (58.2%) showed equal or less staining than in the adjacent epithelium (AKR1C1-). 38% of the HPV-positive tumors were AKR1C1+ and, remarkably also 43% of the HPV-negative tumors were AKR1C1+. Approximately the same percentages were found for AKR1C3 (Table 6.1, 6.2 and Supplementary Table S6.5).

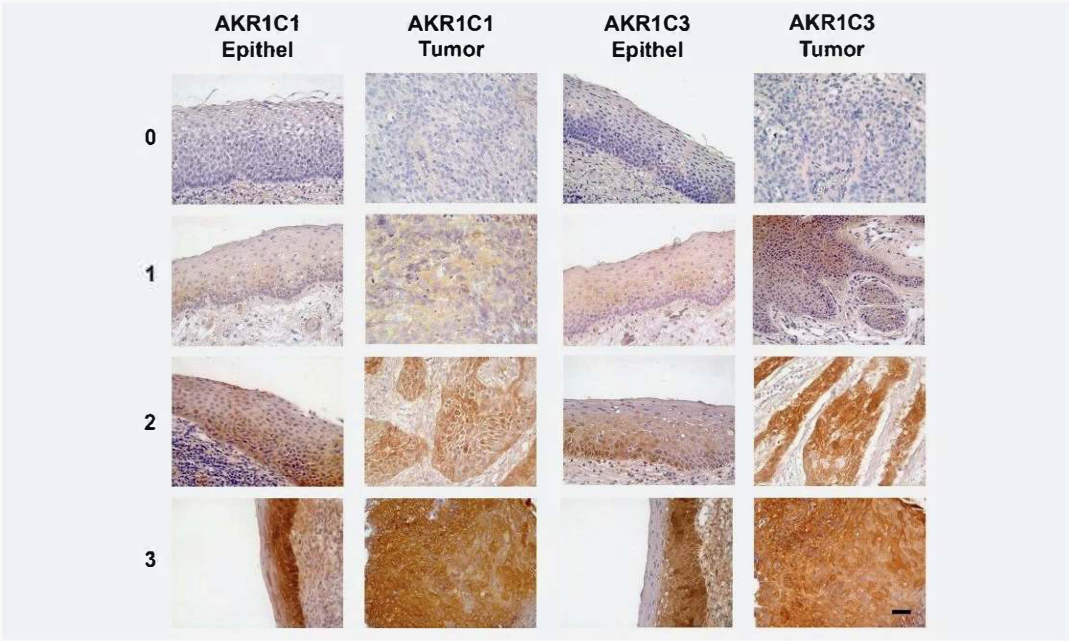

Figure 6.2 Representative immunohistochemical stainings of AKR1C1 and AKR1C3 in non-tumorous and tumor tissue samples. 0 = negative, 1 = faint, 2 = medium, 3 = strong staining. V = 400x.

Table 6.2 Multivariate survival analysis.

				Overall Survival (OS) (multivariate)[1]			Disease-free Survival (DFS) (multivariate)[1]		
Samples analyzed	**Parameters**	**Group**	**No.**	**Hazard ratio**	**95% CI**	**p-Value [2]**	**Hazard ratio**	**95% CI**	**p-Value [2]**
Complete collection									
	All		141						
	AKR1C1	≤ Epithel (-)	82	3.2	1.9 - 5.9	**< 0.0001**	2.7	1.5 - 4.6	**0.001**
		> Epithel (+)	59						
	AKR1C3	≤ Epithel (-)	91	3.7	2.2 - 6.4	**< 0.0001**	3.4	1.9 . 5.8	**< 0.0001**
		> Epithel (+)	50						
	HPV	Positive	48	0.4	0.2 - 0.8	**0.006**	0.4	0.2 - 0.8	**0.007**
		Negative	93						
	T Stage	1-2	78	2.8	1.5 - 5.1	**0.001**	3.2	1.7 - 6.1	**< 0.0001**
		3-4	63						
HPV-positive									
	T stage	HPV+/T1-2	32	1.7	0.6 - 5.4	0.334	2.2	0.7 - 6.6	0.167
		HPV+/T3-4	16						
	N stage	HPV+/N0-1	25	4.1	1.1 - 15.3	**0.037**	2.9	0.9 - 9.7	0.077
		HPV+/N2	23						
	AKR1C1	HPV+/AKR1C1-	30	3.2	0.9 - 10.6	0.063	5.1	1.5 - 22.1	**0.010**
		HPV+/AKR1C1+	18						
	AKR1C3	HPV+/AKR1C3-	31	6.2	1.7 - 23.5	**0.007**	7.5	1.9 - 28.7	**0.003**
		HPV+/AKR1C3+	17						
HPV-negative									
	T stage	HPV-/T1-2	46	2.0	1.1 - 3.9	**0.031**	2.9	1.5 - 5.8	**0.002**
		HPV-/T3-4	47						
	N stage	HPV-/N0-1	37	2.4	1.2 - 4.7	**0.015**	3.1	1.5 - 6.6	**0.001**
		HPV-/N2-3	56						
	AKR1C1	HPV-/AKR1C1-	53	3.2	1.7 - 6.1	**< 0.0001**	2.2	1.2 - 4.1	**0.013**
		HPV-/AKR1C1+	40						
	AKR1C3	HPV-/AKR1C3-	61	3.1	1.7 - 5.8	**< 0.0001**	2.8	1.5 - 5.3	**0.001**
		HPV-/AKR1C3+	32						

[1] Multivariate analysis revealed linear dependency of AKR1C1 with AKR1C3. Therefore, analysis was performed individually and data from both analyses are presented;.[2] p-Value calculated by log-rank (Mantel-Cox)test, multivariate. Bold: significant values ≤ 0.050.

Subgroup analysis of AKR1C stained samples for 3-NT and NRF2

To validate the association between mRNA array data and oxidative stress, we immunostained n=24 randomly chosen HPV-positive and -negative samples from cohort II against 3 Nitrotyrosine (3-NT), which is generated by Peroxynitrite and the oxidative stress related transcription factor Nuclear Factor (Erythroid-derived 2)-Like 2 (NF2L2 / NRF2)[19,20] (Supplementary Figure S6.5). In HPV-positive samples, both 3-NT and NRF2 showed similar staining intensities compared to AKR1C3 staining ($p<0.0001$) (Supplementary Figure S6.5 and Supplementary Table S6.6). In HPV-negative samples some exceptions were found, i.e. one discordant staining pattern for 3-NT and two for NRF2 in comparison with AKR1C3 (3-NT $p=0.011$; NRF2 $p=0.197$).

Subgroup analysis of AKR1C stained samples for viral integration

In order to examine if the AKR1C1+ / C3+ tumors in the HPV-positive group also show a higher number of cases with viral integration, we studied 20 cases of the HPV-positive OPSCC from cohort II of which sufficient fresh frozen tissue was available for viral integration analysis. APOT-PCR showed 7 cases with integration, 4 with a mixed pattern, and 9 with episomal viral DNA. Five of seven OPSCC with integrated virus were AKR1C1+ / C3+, whereas all 9 cases with episomal virus were negative. This is in line with the results from cohort I. The cases with mixed viral physical status showed both positive (n=3) and negative (n=1) staining.

Correlation of AKR1C1 and AKR1C3 with clinicopathological data

By correlating the immunohistochemical results with clinicopathological data we demonstrated a highly significant association of AKR1C1+ and AKR1C3+ tumors with tumor relapse and patient death ($p<0.0001$) (Table 6.2 and Supplementary Table S6.7). Although, there is a strong association for both proteins with HPV-status, this is less evident for history of alcohol (AKR1C1 $p=0.093$, AKR1C3 $p=0.044$) and tobacco (AKR1C1 $p=0.280$, AKR1C3 0.093) consumption (Table 6.1). Furthermore, HPV-status was associated with known parameters such as a lower T-stage, less tumor relapse and death (Table 6.2 and Supplementary Table S6.7).

AKR1C1 / C3 protein expression, HPV and survival in OPSCC

AKR1C1 / C3 protein expression did correlate with both worse overall (OS) (Figure 6.3) and disease-free survival (DFS) rates ($p<0.0001$ in all cases) as did HPV-status ($p<0.0001$ in both cases) and low T staging ($p=0.001$) and lacking smoking history ($p=0.039$) (Table 6.2 Supplementary Table S6.7 and Supplementary Figure S6.7).

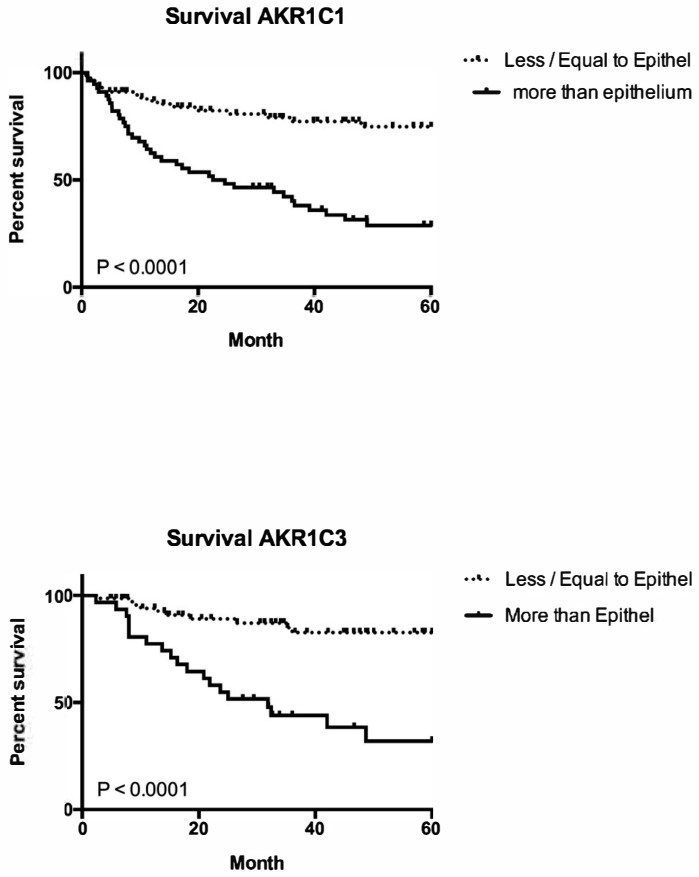

Figure 6.3 Univariate survival analysis for AKR1C1 (A) and AKR1C3 (B) expression status. Kaplan-Meier plot for overall survival (OS) in patients with low vs. high protein expression. P value was derived by log-rank/Mantel-Cox test.

We also correlated separately AKR1C1 and AKR1C3 staining in the tumor and in the adjacent epithelium in relation to survival. This revealed that particularly low expression in the tumor adjacent epithelium is already associated with unfavorable prognosis (AKR1C1 p=0.0104; AKR1C3 p=0.0245), and in that combination with higher expression in the adjacent tumor, this association becomes more prominent (p<0.0001 for both AKR1C1 and AKR1C3) (Figure 6.3, Supplementary Figure S6.6, Table 6.2, Supplementary Table S6.5 and Supplementary Table S6.7).

Interestingly, in the subgroup of patients with HPV-positive OPSCC, AKR1C1+ and C3+ significantly indicated a poor prognosis (AKR1C1 median OS 47.5 vs. 95.4 month, median DFS 38.9 vs. 90.0 month; AKR1C3 median OS 41.7 vs. 98.6 month, median DFS 35.3 vs. 90.3 month; all p≤0.001). Similarly, in the subgroup of patients with HPV-negative tumors, AKR1C1+ / C3+ significantly indicated an unfavorable prognosis (AKR1C1 median OS 40.0 vs. 89.2 month, median DFS 28.3 vs. 58.7 month; AKR1C3 median OS 37.1 vs.

84.0 month, median DFS 24.8 vs. 59.0; all p<0.0001) (Table 6.2 Supplementary Table S6.7 and Supplementary Figure S6.7).

AKR1C1 / C3 protein expression and treatment modalities

Since the AKR1C enzymes are reported to be Phase I detoxifying enzymes especially involved in the turnover of platin drugs, we correlated AKR1C staining intensities with treatment modalities (Table 6.2 Supplementary Table S6.7 and Supplementary Figure S6.7). Consequently, AKR1C1+ / C3+ turned out to be a negative predictor in correlation to Chemo- and Radiotherapy both in OS and DSF. Most prominent, low AKR1C1 and AKR1C3 expression turned out to be a highly significant predictors for favorable outcome in surgical treatment (AKR1C1 OS 85.2%, DFS 85.2%; AKR1C3 OS 86.2%, DFS 85.1%; all p<0.0001). In multivariate analysis, we included all predictors being significant in univariate analysis (Table 6.2 and Supplementary Figure S6.7) for OS and DFS. T-stage, HPV and AKR1C3 turned out to be independent significant prognostic predictors.

Conclusion

It is still unclear if HPV integration in OPSCC affects the levels of viral and/or HPV-disrupted human gene transcripts. In previous work, we did not find clear evidence for this hypothesis[3,21], since constitutive, rather than a high level of viral and virally disrupted gene transcripts was observed in the tumors. Therefore, in this study it was our aim to perform a genome-wide screen to identify human genes which expression is influenced by integration of HPV16. By comparing HPV16-positive OPSCC harboring episomal or integrated virus using mRNA microarray expression profiling, we identified a unique signature of differentially expressed human mRNAs in relation to viral physical state. Upregulated AKR1C1 and AKR1C3 expression was observed in HPV16-positive OPSCC with viral integration and, interestingly, associated with poor prognosis both in HPV-positive and -negative tumors.

Despite some literature reporting that in OPSCC with integrated HPV there may be a direct effect of viral protein expression on the host genome and human gene expression[9,22], in most tumors this effect is unclear. Here we provide new data showing expression signatures associated with HPV16 physical status in OPSCC. Interestingly, the tumors with viral integration showed deregulated expression of genes involved in metabolic pathways (Figure 6.1B), frequently including upregulated AKR1C1 and/or AKR1C3 expression. Furthermore, this upregulated expression was also observed in a subgroup of HPV-negative OPSCC (see further below). Increased expression of AKR1C genes has previously been reported by others. Martinez *et al.* compared gene expression

in a small group of HPV-positive vs. -negative OPSCC by mRNA expression profiling, finding higher expression of AKR1C3 in HPV-negative tumors.[23] In a protein expression study on uterine cervical cancer (n=145), Ueda *et al.* found a significant correlation between AKR1C1 (termed by alternative nomenclature as DDH1, Dihydrodiol Dehydrogenase Type I) and tumors infected with HPV types 16 and 18, FIGO stage, lymph node involvement and patients' survival.[24] AKR1C1 and AKR1C3 upregulation has also been observed after transfection of the HPV-negative uterine cervical carcinoma cell line C33A with a HPV16-E6*I construct.[25] The translated truncated HPV16-E6 product E6*I appeared to have a direct promoting effect on AKR1C1 and AKR1C3 expression via the SP1 binding sites in their promotor regions.

Our observation that the AKR1C1 and AKR1C3 genes are upregulated in OPSCC with integrated HPV16 do fit with these experiments, since we and others also detected E6*I mRNA upregulation in tumors with integrated HPV16.[26,27] Like tobacco smoke, E6*I can promote reactive oxygen species (ROS) activated pathways, including activation of NRF2[16]. Known consequences of NRF2 activation are e.g. activated PI3K-AKT signaling, deficiency in autophagy, impaired DNA damage response, enhanced cell proliferation, and interestingly, metabolic reprogramming and chemotherapeutic drug modification resulting in resistance[17,28,29] (Figure 6.4).

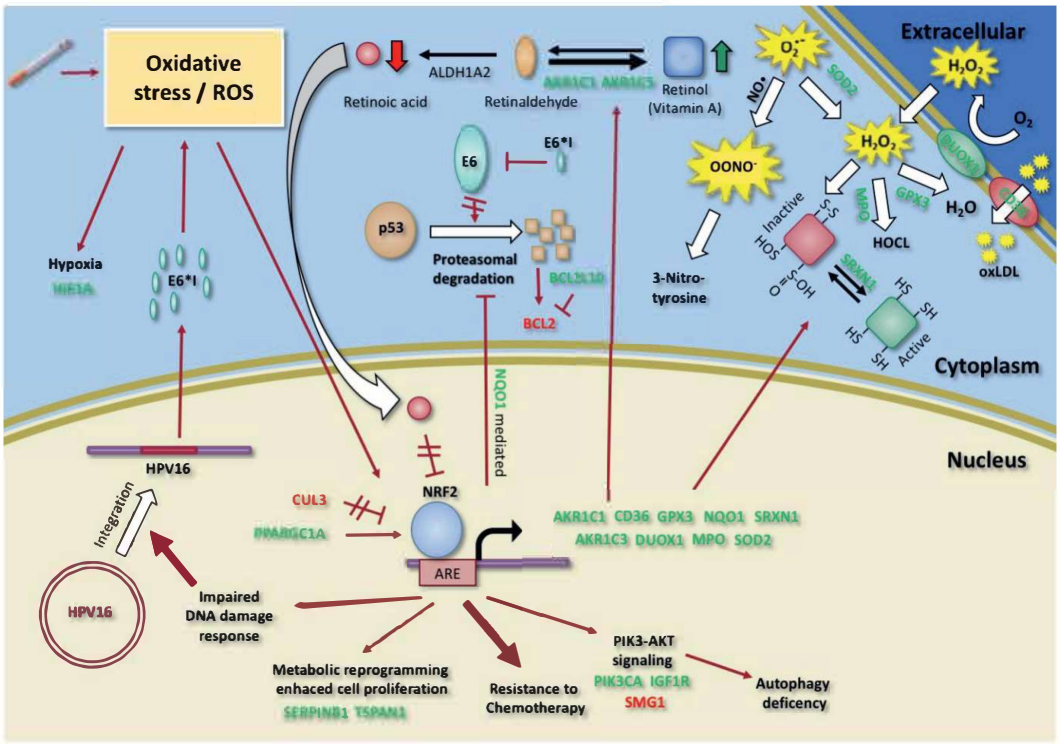

Figure 6.4 Supposed mechanisms combining the effects of HPV16-positive OPSCC with viral genome integration and HPV-negative / Smoking-positive OPSCC.

Host genome integration of HPV16 leads to upregulation of the viral E6 spliced isoform E6*I. Like tobacco smoke, E6*I can promote reactive oxygen species (ROS) signaling by influencing transcriptional factor expression like NRF2 (Nuclear Factor Like 2) to modulate the expression of antioxidant enzymes including AKR1C1 and AKR1C3.[25] The AKR1Cs 1 and 3 are known to catalyze the production of retinol isoforms from retinaldehyde and subsequently lowering retinoic acid concentrations.[37] Retinoic acid is a known inhibitor of NRF2 function, therefore decreased concentrations of retinoic acid lead to additional NRF2 activation.[32] Furthermore, NRF2 controlled NQO1 (NAD(P)H dehydrogenase [quinone] 1) stabilizes p53, preventing it from degradation.[47] Several additional NRF2 activated oxidative stress response genes harboring ARE elements were found to be upregulated by our mRNA array analysis including CD36 (cluster of differentiation 36, also known as fatty acid translocase), DUOX1 (Dual Oxidase 1), GPX3 (Glutathione Peroxidase 3), MPO (Myeloperoxidase), SOD2 (Superoxide Dismutase 2), and SRXN1 (Sulfiredoxin 1).[17,48,49] In HPV16 integration positive OPSCC, HPV16-E6 might furthermore be blocked by its isoform E6*I.[5] As a consequence, we found BCL2 (B-cell lymphoma 2) to be downregulated. Through increased expression of its antagonist BCL2L10, putative positive effects on apoptosis and autophagy might be directly antagonized.
Known consequences of NRF2 activation are e.g. activated PIK3-AKT signaling, metabolic reprogramming, enhanced cell proliferation, deficiency in autophagy, and interestingly, resistance to chemotherapy as well as impaired DNA damage response. The latter is supposed to promote integration of HPV16 episomes into the human genome.Green = upregulation, red = downregulation in mRNA array analysis.

AKR1C1 and AKR1C3 belong to the aldo-keto reductase (AKR) superfamily of NAD(P)H dependent oxidoreductases. Both enzymes have multiple cellular functions in e.g. regulating prostaglandin, steroid hormone and retinoid metabolism and moreover are Phase I detoxifying enzymes involved in modifying toxic substances, such as chemotherapeutic drugs and tobacco smoke components.[17,30,31] Besides direct upregulation by E6*I, the expression of both enzymes can be initiated under the presence of ROS by the Keap1/Cul3/Nrf2 system via direct binding of NRF2 to the antioxidant response element (ARE) located in the promotor regions of the AKR1Cs and in several additional oxidative stress related genes which proved to be deregulated in this study (Figure 6.4). Interestingly, AKR1C1 and AKR1C3 generate several feedback loops controlling their own expression. On the one hand, through the production of retinol from retinaldehyde, so that the concentration of retinoic acid, a known inhibitor of NRF2 function, is lowered, which leads to additional NRF2 activation.[32] On the other hand, in response to tobacco smoke, they exacerbate the carcinogenicity of polycyclic aromatic hydrocarbons (PAH) by oxidizing *trans*-hydrodiol functional groups (leading to e.g. benzo[*a*]pyrene-7,8-dione [BPQ]), which in turn binds AREs and further stimulates AKR1C1 and AKR1C3 expression. In this way PAH *trans*-hydrodiols would enhance their own genotoxicity by inducing expression of the AKR1Cs.[17] Because tobacco smoke thus can result in upregulation of AKR1C1 and AKR1C3 and 86% of patients in our cohort of 141 OPSCC were smokers (cohort II), we assessed if there was an association between these parameters by analyzing expression in the tumors with their adjacent epithelium. Interestingly, only in the adjacent epithelium there was prognostic difference between

high and low stained samples (Supplementary Figure S6.6). This prognostic effect even became higher when the normal epithelium staining intensity was at least one grade higher than the tumor staining intensity. From these data and expression of NRF2 and 3-NT (Supplementary Figure S6.5 and Supplementary Table S6.6, Figure 6.4) we conclude that high staining in the epithelium is an indicator for a better capacity to handle oxidative stress. As a consequence, higher staining in the tumor than in the epithelium is overwhelming this individual capacity to deal with oxidative stress resulting in a more aggressive tumor. Our approach of semi-quantitative normalization of tumor staining intensity by comparing it with adjacent epithelium might be furthermore supported by a study from Namani et al., where RNAseq data from the TCGA HNSCC cohort were normalized against normal tissue expression and subsequently lead to a signature of overexpressed oxidative stress response genes including AKR1C1 and AKR1C3.[33] For confirmation, we analyzed RNAseq data from the TCGA HNSCC cohort with both tumor and corresponding normal tissue expression data available (n=43). Again, overexpression of AKR1C1 and AKR1C3 in the tumor compared to corresponding normal tissue correlated with unfavorable survival (AKR1C1 p=0.0406; AKR1C3 p=0.0232) (data not shown).

The presence of a differential expression of AKR1C1 and AKR1C3 between tumor and epithelium might be an optimal readout for this metabolic reprogramming pattern.[17,34] We found no correlation between smoking and upregulation of AKR1C1 and AKR1C3 expression. An additional mechanism promoting NRF2 and subsequently AKR1C1 and AKR1C3 activation is through methylation and mutation of genes coding for proteins of the oxidative stress system (e.g. CUL3, KEAP1, NRF2, RBX1), especially found in HPV-negative HNSCC.[34.35] Consequently, in HPV-negative samples we found 18.2% cases with discordant NRF2 staining intensities compared to AKR1C staining (Supplementary Table S6.6). Interestingly, in an independent study comparing methylation profiles of HPV-positive and -negative OPSCC, methylation of the ALDH1A2 gene and subsequent downregulation of gene expression also was related to retinol production, activation of oxidative stress response and an unfavorable prognosis[36,37] (Figure 6.4). This is in agreement with our findings presented here.

By univariate analysis we found several parameters to be associated with unfavorable prognosis in OPSCC, including upregulation of AKR1C1 and AKR1C3 along with known factors such as HPV, T-stage, N-stage and smoking. HPV, T-stage, AKR1C1 and AKR1C3 turned out to be significant also in multivariate analysis. Moreover, AKR1C1 and AKR1C3 expression turned out to be strong indicators of prognosis in both HPV-positive and –negative OPSCC. Interestingly, smoking proved not to be an independent variable in multivariate analysis, which might be explained by the fact that AKR1C1 and AKR1C3 are indicators of oxidative stress including, but not restricted to tobacco smoke

components.[31] If these results could be confirmed in larger studies, than AKR1C1 and/or AKR1C3 may be considered to be included in prediction models in OPSCC and HNSCC.[38-40] Low risk groups can then profit from de-intensification of treatment protocols[41], whereas intermediate and high risk groups could be selected for other therapeutic options, such as inhibitors of the PI3K and NRF2 pathways including the AKR1C proteins.[17,42,43]

Potential treatment options addressing oxidative stress related pathways involving NRF2, AKR1C and PI3K upregulation were recently discussed.[44-46] However, due to the high expression of AKR1C enzymes in normal tissues like liver and pancreas, it will be important to investigate potential adverse events of these drugs in these tissues.

In conclusion, we provide further evidence that viral host genome integration of HPV16 has a biological effect on the host cell, e. g. by deregulating metabolic pathways including upregulating AKR1C1 and AKR1C3 expression. Increased expression of these proteins was also found to have impact on prognosis in HPV-negative tumors. Therefore, our findings may impact prognosis as well as increase options for treatment of OPSCC in general.

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Supplementary data

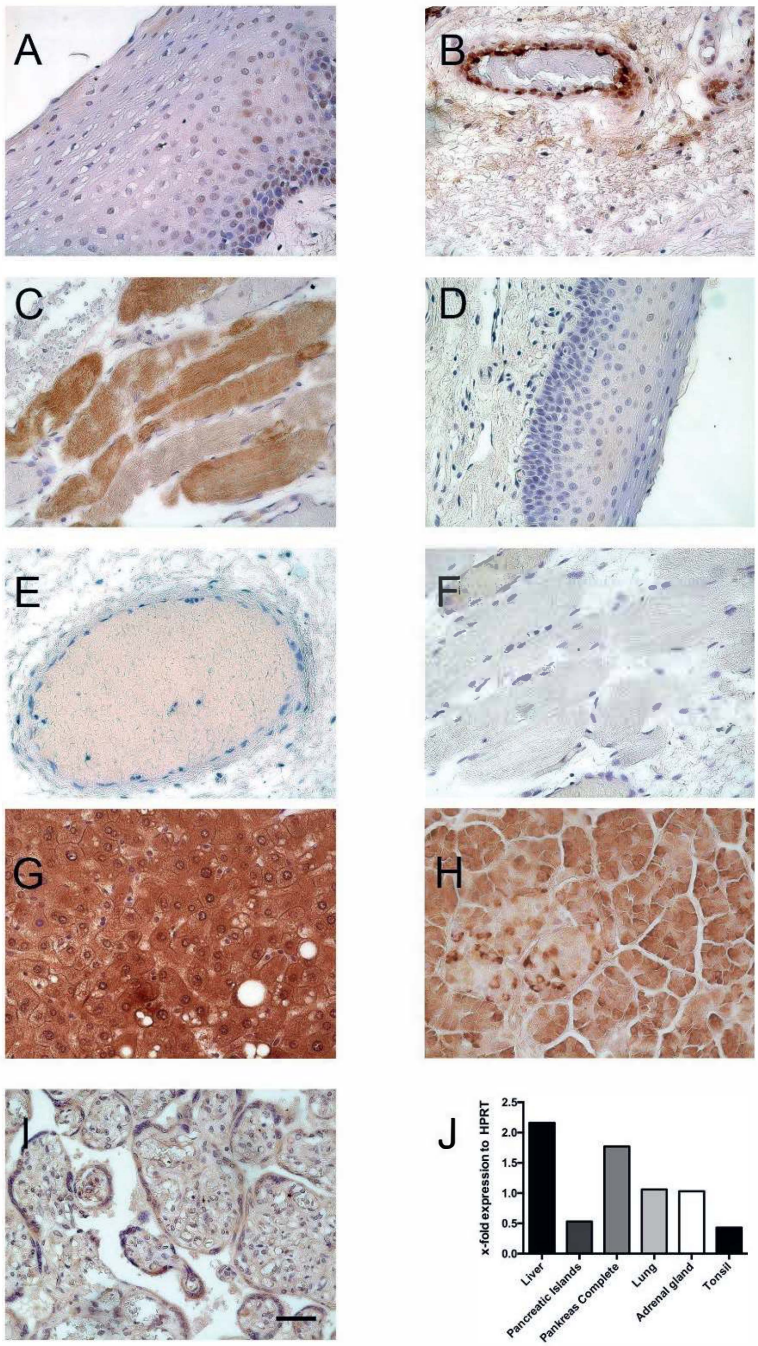


Figure S6.1 Expression analysis of AKR1C1 in normal control Tissue. (A-C) Representative samples of tissue sections from normal tonsils. (A) Epithelium, (B) Endothel, (C) Muscle. (D-F) Control staining of normal tonsils without primary antibody. (D) Epithelium, (E) Endothel, (F) Muscle. (G-I) Control staining of selected tissue controls. (G) Liver, (H) Pancreas and (I) Placenta. V = 400×. (J) RT-qPCR analysis of mRNA expression in control tissue as indicated.

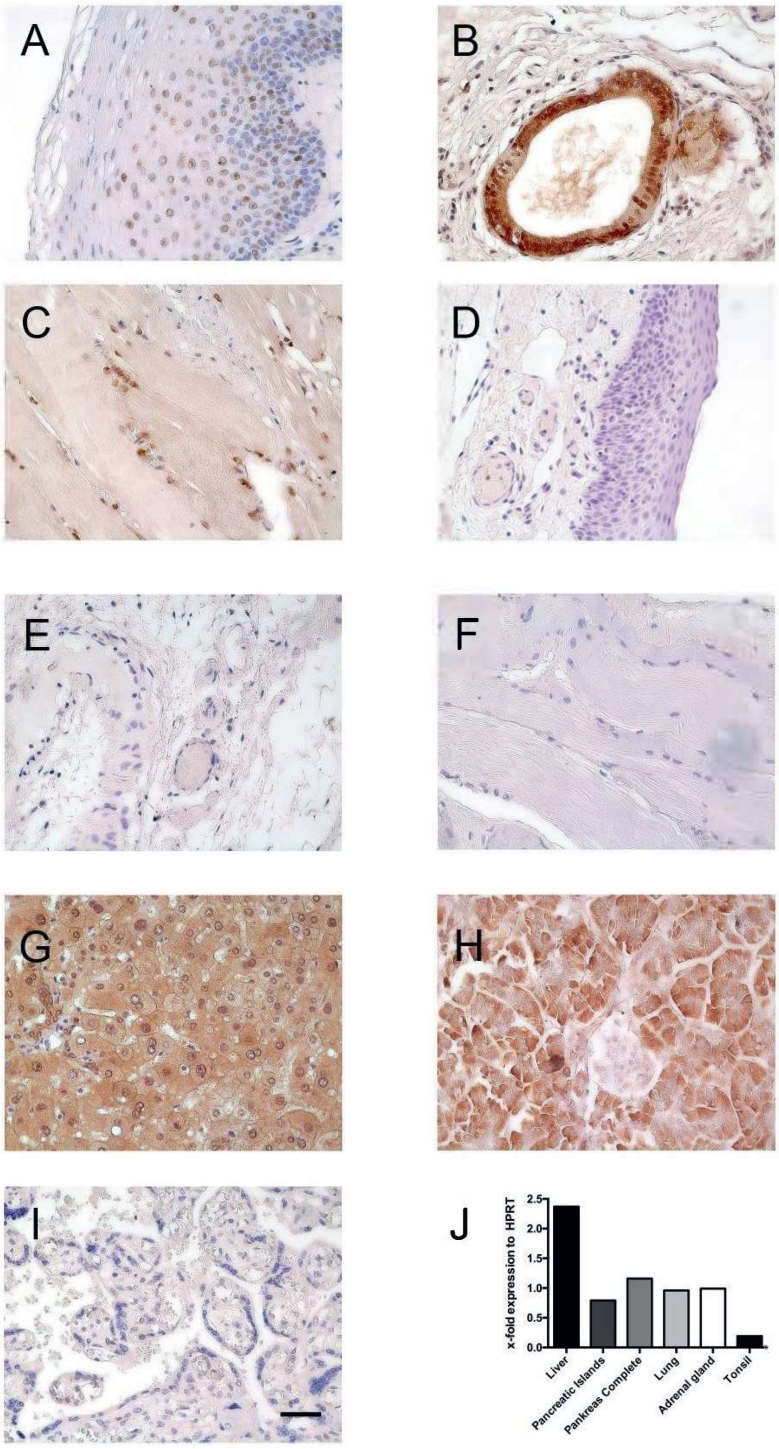


Figure S6.2 Expression analysis of AKR1C3 in normal control Tissue. (A-C) Representative samples of tissue sections from normal tonsils. (A) Epithelium, (B) Endothel, (C) Muscle. (D-F) Control staining of normal tonsils without primary antibody. (D) Epithelium, (E) Endothel, (F) Muscle. (G-I) Control staining of selected tissue controls. (G) Liver, (H) Pancreas and (I) Placenta. V = 400x. (J) RT-qPCR analysis of mRNA expression in control tissue as indicated.

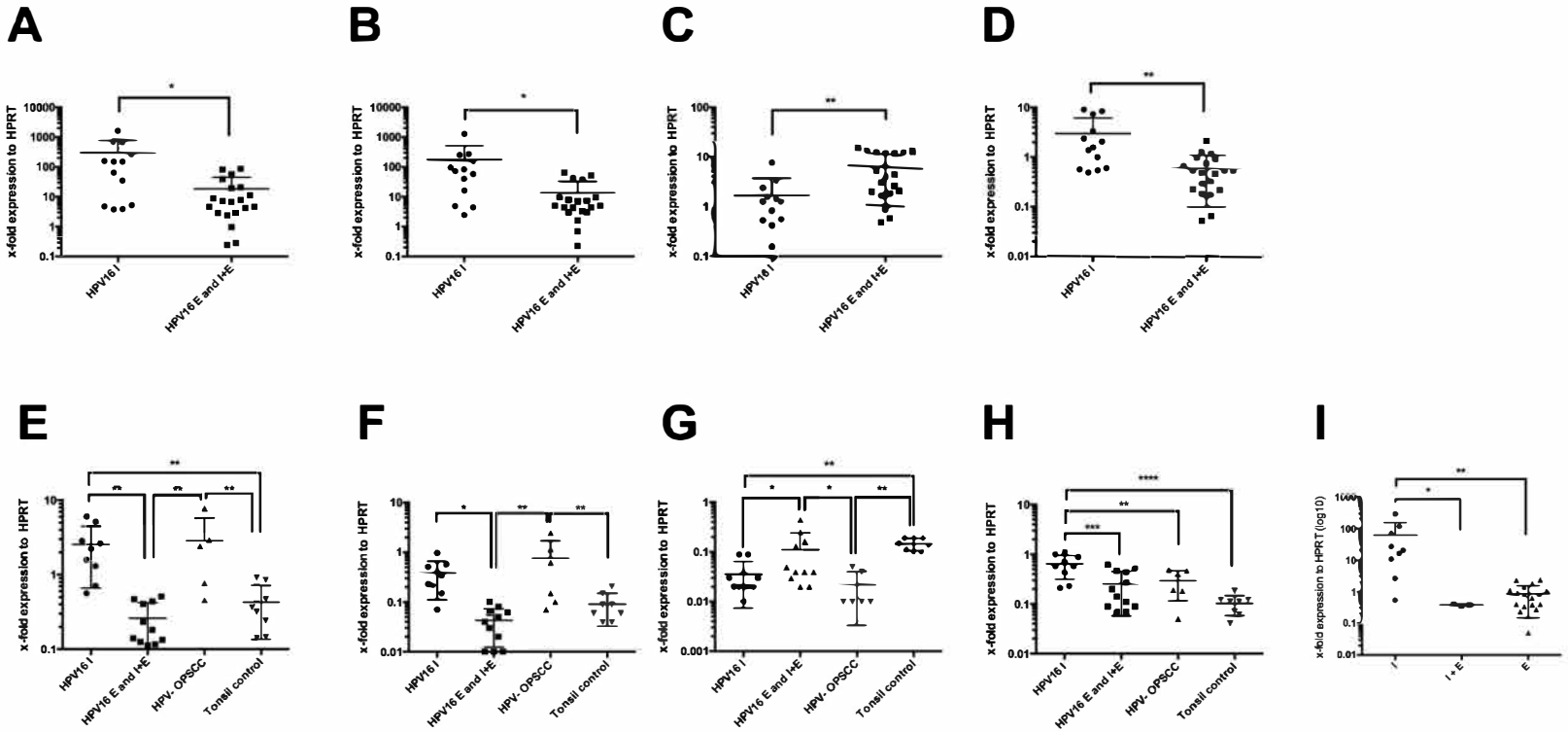


Figure S6.3 mRNA expression analysis of selected gene products identified by gene ontology pathway analysis. (A, E) AKR1C1, (B, F) AKR1C3, (C, G) BCL2, (D, H) BCL2L10. (A - D) Expression data obtained by microarray were extracted and normalized to HPRT. (E - H) mRNA levels determined by RT-qPCR to confirm microarray data. HPV-negative Tumor tissue and tissue of non-tumorous tonsils served as controls. Bars: Mean with standard deviation. (I) Expression of HPV16-E6*I in Cohort I. I vs. I+E p=0.0131; I vs. E p=0.0010.

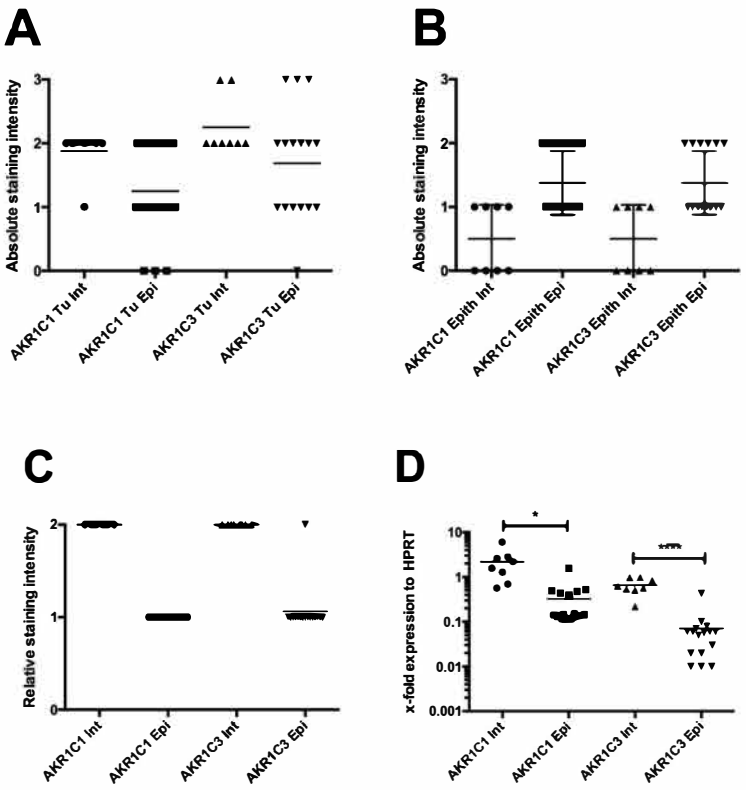
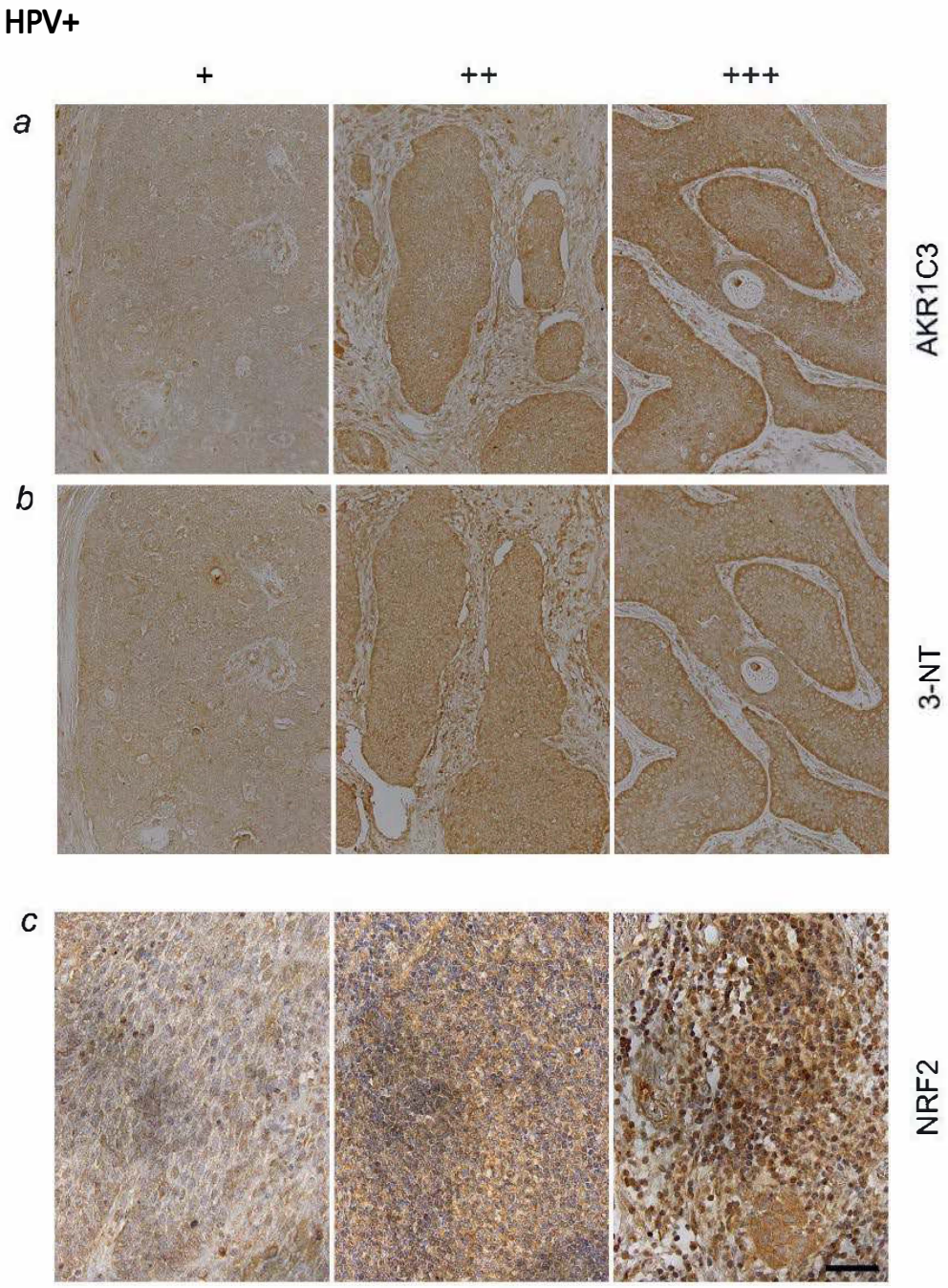


Figure S6.4 Scoring of tumor samples from cohort 1 (n = 24). AKR1C1 and AKR1C3 Immunohistochemical staining is scored in relation to HPV physical status and compared to RT-qPCR data of same tumors. (A) Absolute scoring of tumor cells. 0 = no staining, 1 = weak staining, 2 = medium staining. (B) Absolute scoring of epithelial cells. 0 = no staining, 1 = weak staining, 2 = medium staining. (C) Relative staining of tumor cells compared to adjacent normal epithelium. 0 = weaker than epithelium, 1 = equal to epithelium, 2 = stronger than epithelium. (D) Corresponding RT-qPCR data. Bars = Mean. (*) $p<0.05$, (****) $p<0.00001$.


HPV+
+
++
+++
a
b
c
AKR1C3
3-NT
NRF2

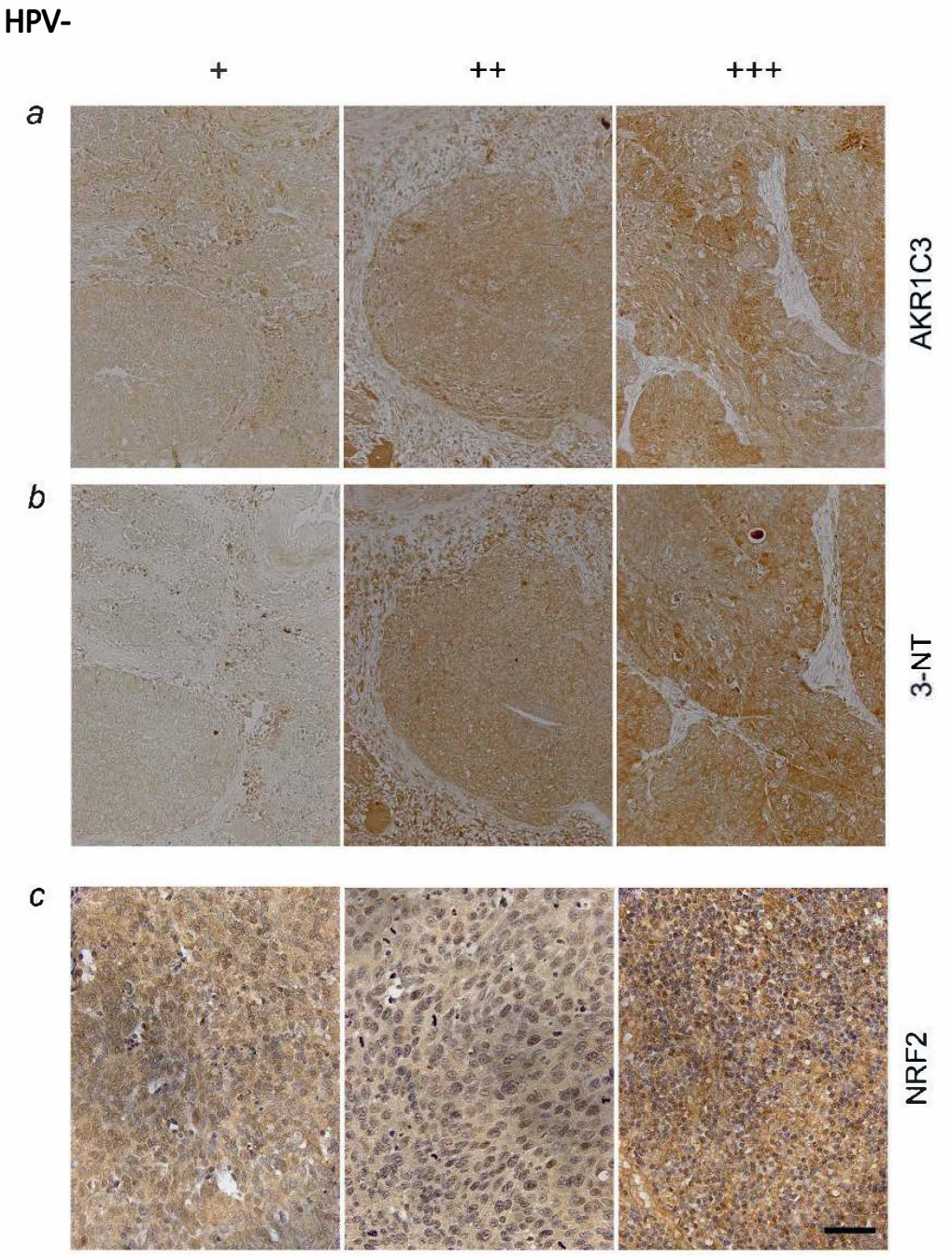
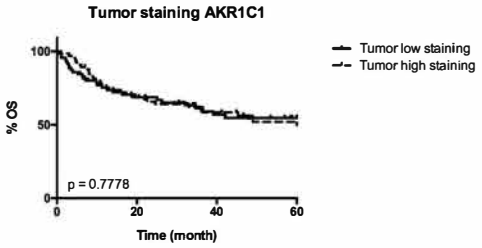
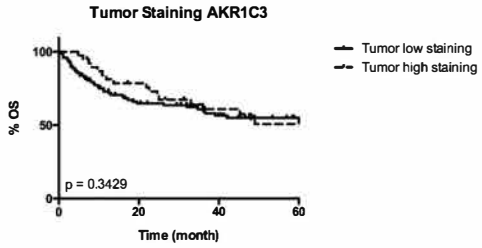
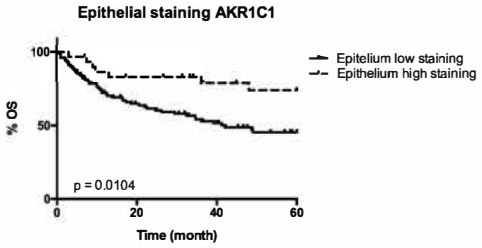
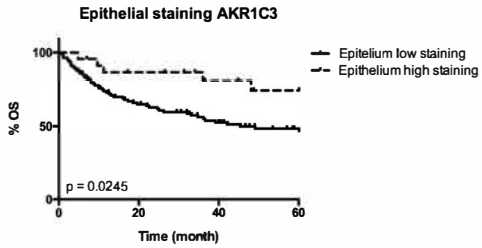


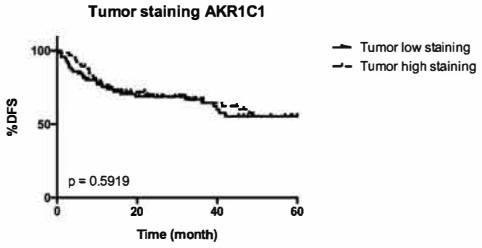
Figure S6.5 Expression analysis of AKR1C3, 3-Nitrotyrosine and NRF2 in HPV-positive and -negative tumor tissue. (A) Representative samples of AKR1C3 immunohistochemical staining in tumor tissue sections. (B) 3-Nitrotyrosine (3-NT) staining in a consecutive slide. (C) NRF2 staining from the same patient sample as A and B at higher magnification showing representative nuclear staining. (A) and (B) V = 400×, (C) V = 630x.

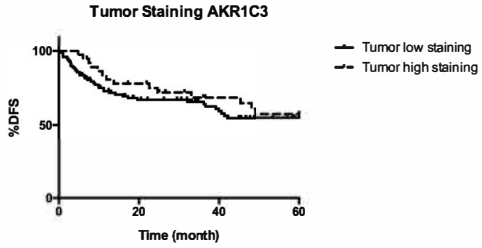

Tumor staining AKR1C1
Tumor low staining
Tumor high staining
% OS
100
50
0
p = 0.7778
0
20
40
60
Time (month)

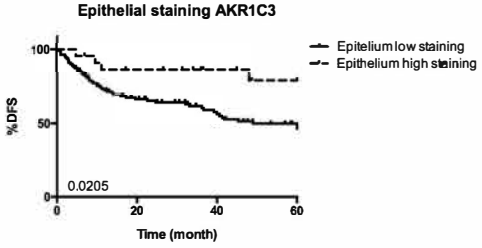

Tumor Staining AKR1C3
Tumor low staining
Tumor high staining
% OS
100
50
0
p = 0.3429
0
20
40
60
Time (month)

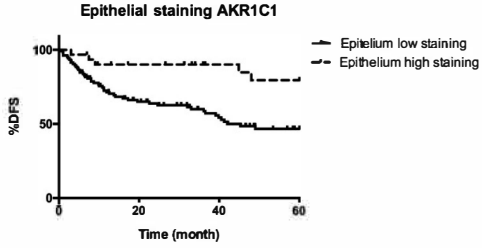

Epithelial staining AKR1C1
Epitelium low staining
Epithelium high staining
% OS
100
50
0
p = 0.0104
0
20
40
60
Time (month)


Epithelial staining AKR1C3
Epitelium low staining
Epithelium high staining
% OS
100
50
0
p = 0.0245
0
20
40
60
Time (month)


Tumor staining AKR1C1
Tumor low staining
Tumor high staining
%DFS
100
50
0
p = 0.5919
0
20
40
60
Time (month)


Tumor Staining AKR1C3
Tumor low staining
Tumor high staining
%DFS
100
50
0
0
20
40
60
Time (month)


Epithelial staining AKR1C3
Epitelium low staining
Epithelium high staining
%DFS
100
50
0
0.0205
0
20
40
60
Time (month)


Epithelial staining AKR1C1
Epitelium low staining
Epithelium high staining
%DFS
100
50
0
0
20
40
60
Time (month)

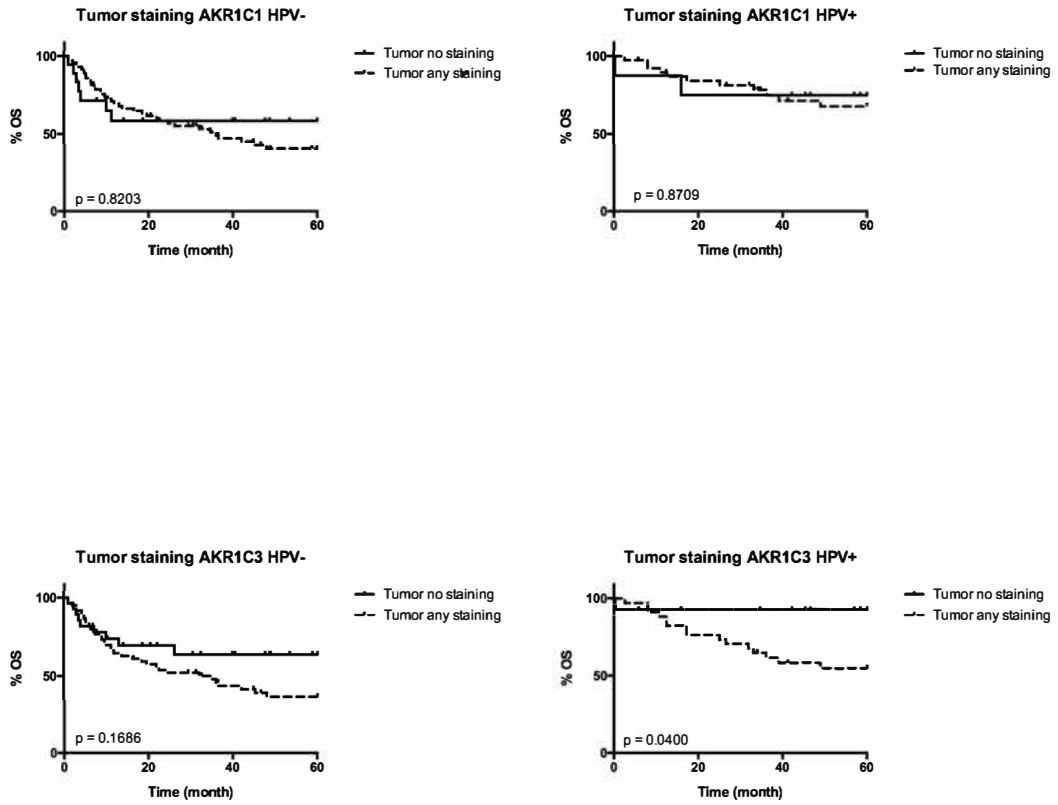
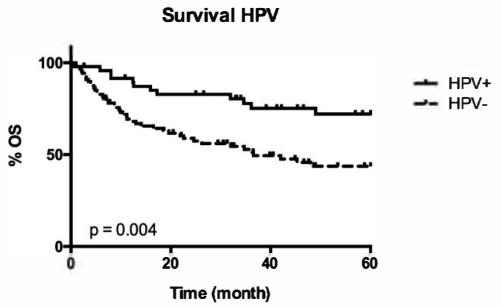
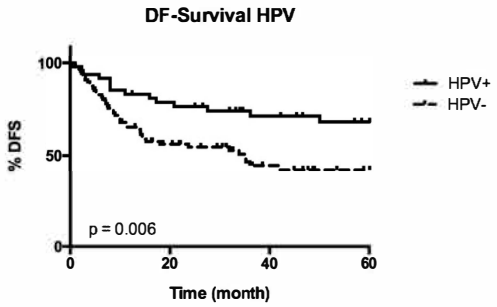
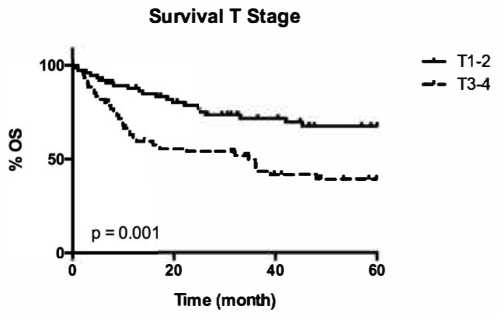
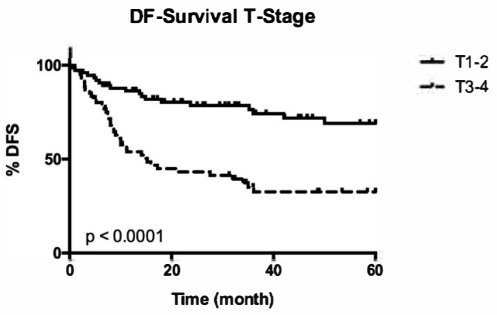


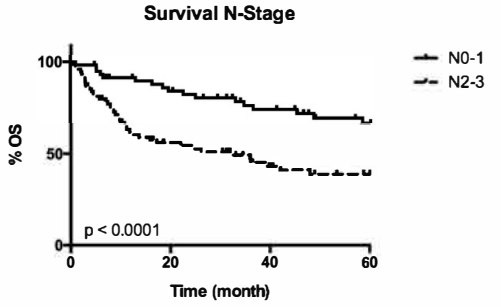
Figure S6.6 Combined univariate survival analysis for AKR1C1 and AKR1C3 expression status separated for expression intensities in tumor tissue and adjacent normal epithelium. Kaplan-Meier plot for overall survival (OS) and disease free survival (DFS) in patients with low vs. high AKR1C1 and AKR1C3 protein expression. P value was derived by log-rank/Mantel-Cox test.

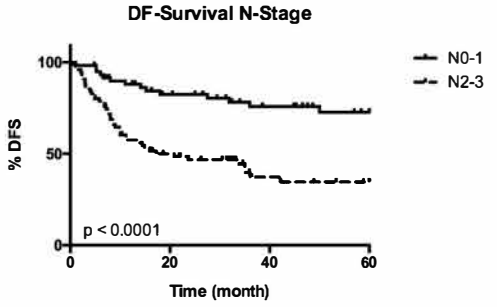

Survival HPV
% OS
100
50
0
p = 0.004
0
20
40
60
Time (month)
HPV+
HPV-

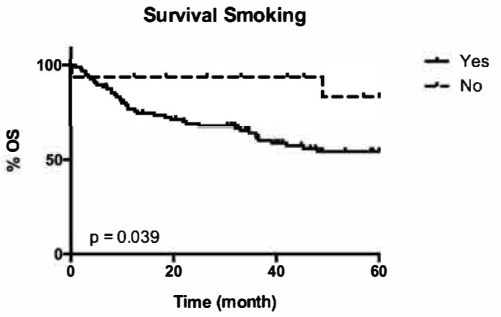

DF-Survival HPV
% DFS
100
50
0
p = 0.006
0
20
40
60
Time (month)
HPV+
HPV-

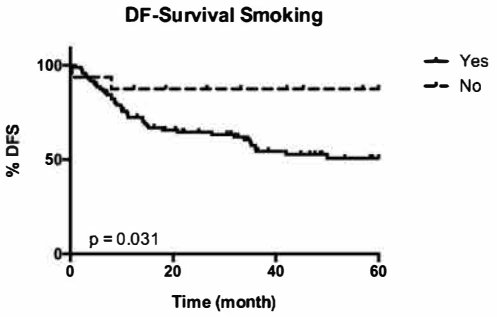

Survival T Stage
% OS
100
50
0
p = 0.001
0
20
40
60
Time (month)
T1-2
T3-4

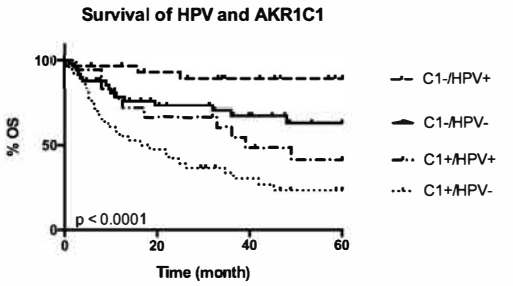

DF-Survival T-Stage
% DFS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
T1-2
T3-4

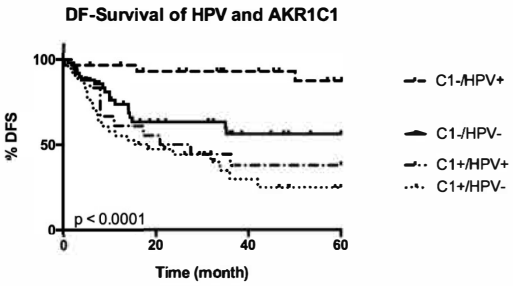

Survival N-Stage
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
N0-1
N2-3

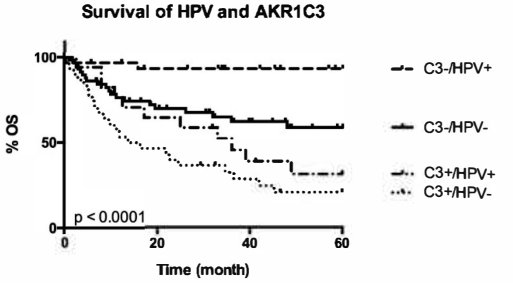

DF-Survival N-Stage
% DFS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
N0-1
N2-3

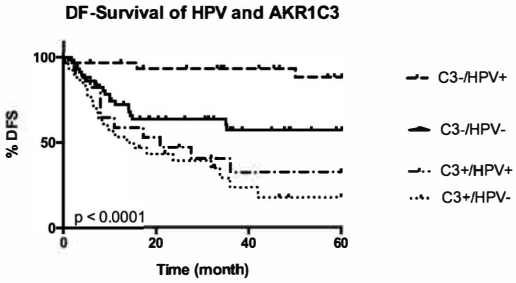

Survival Smoking
% OS
100
50
0
p = 0.039
0
20
40
60
Time (month)
Yes
No

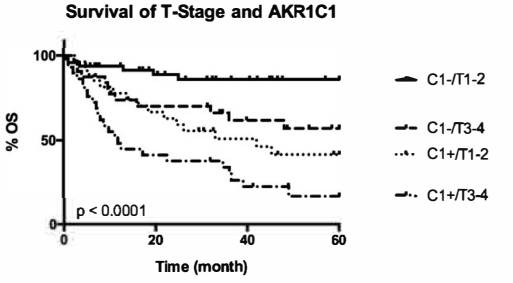

DF-Survival Smoking
% DFS
100
50
0
p = 0.031
0
20
40
60
Time (month)
Yes
No

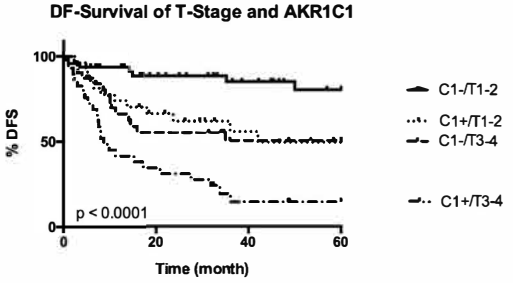

Survival of HPV and AKR1C1
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/HPV+
C1-/HPV-
C1+/HPV+
C1+/HPV-

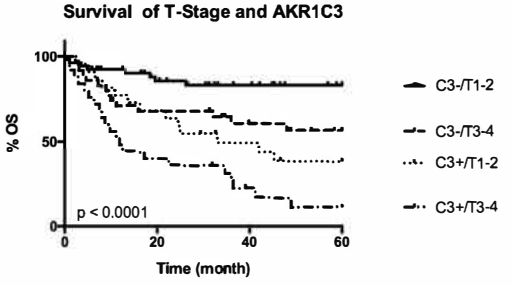

DF-Survival of HPV and AKR1C1
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/HPV+
C1-/HPV-
C1+/HPV+
C1+/HPV-

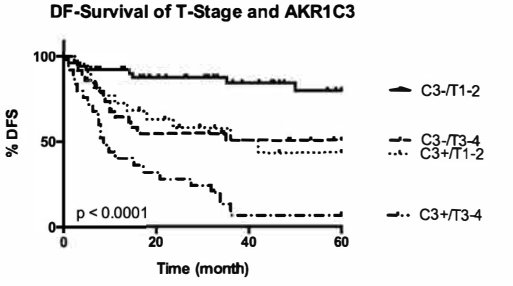

Survival of HPV and AKR1C3
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/HPV+
C3-/HPV-
C3+/HPV+
C3+/HPV-

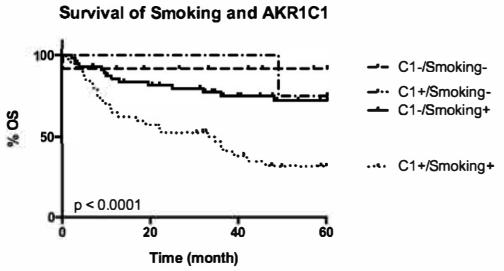

DF-Survival of HPV and AKR1C3
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/HPV+
C3-/HPV-
C3+/HPV+
C3+/HPV-

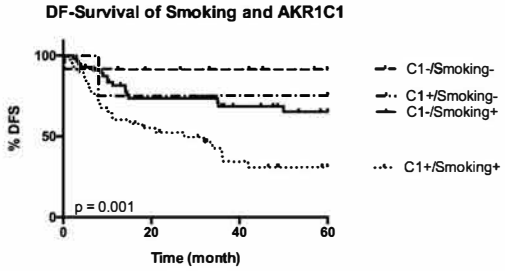

Survival of T-Stage and AKR1C1
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/T1-2
C1-/T3-4
C1+/T1-2
C1+/T3-4

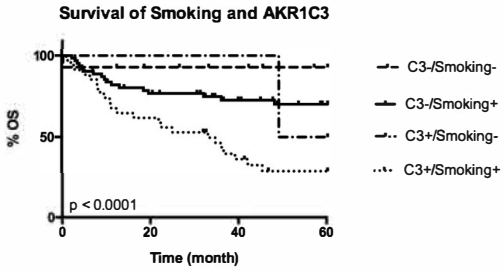

DF-Survival of T-Stage and AKR1C1
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/T1-2
C1+/T1-2
C1-/T3-4
C1+/T3-4

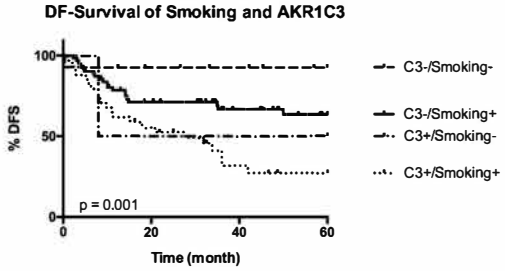

Survival of T-Stage and AKR1C3
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/T1-2
C3-/T3-4
C3+/T1-2
C3+/T3-4

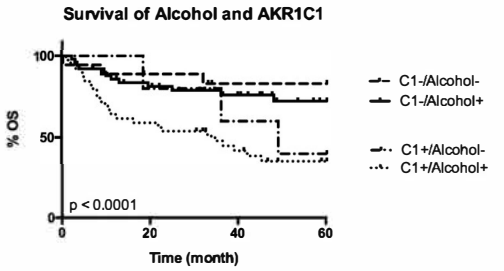

DF-Survival of T-Stage and AKR1C3
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/T1-2
C3-/T3-4
C3+/T1-2
C3+/T3-4

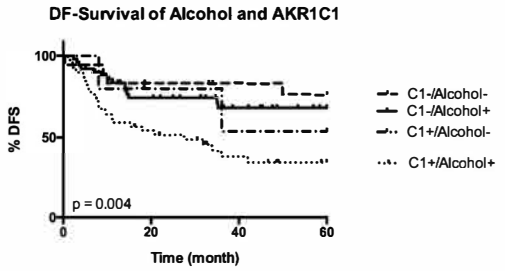

Survival of Smoking and AKR1C1
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/Smoking-
C1+/Smoking-
C1-/Smoking+
C1+/Smoking+

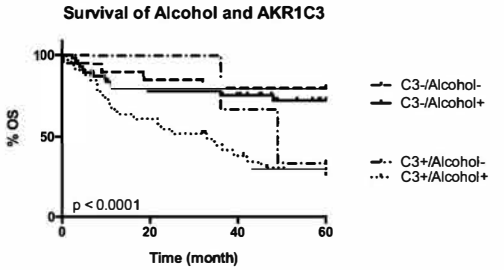

DF-Survival of Smoking and AKR1C1
% DFS
100
50
0
p = 0.001
0
20
40
60
Time (month)
C1-/Smoking-
C1+/Smoking-
C1-/Smoking+
C1+/Smoking+

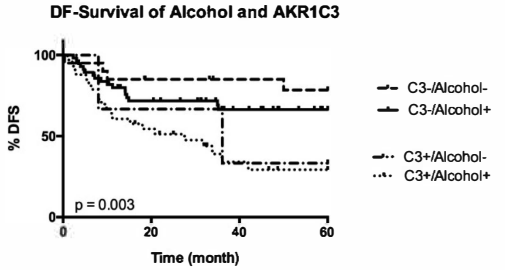

Survival of Smoking and AKR1C3
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C3-/Smoking-
C3-/Smoking+
C3+/Smoking-
C3+/Smoking+

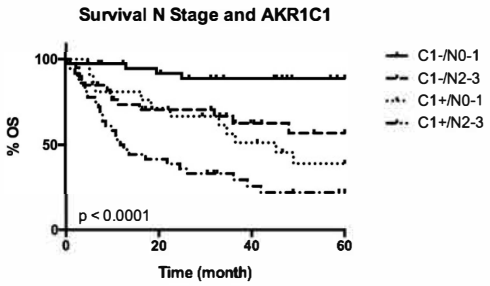

DF-Survival of Smoking and AKR1C3
% DFS
100
50
0
p = 0.001
0
20
40
60
Time (month)
C3-/Smoking-
C3-/Smoking+
C3+/Smoking-
C3+/Smoking+

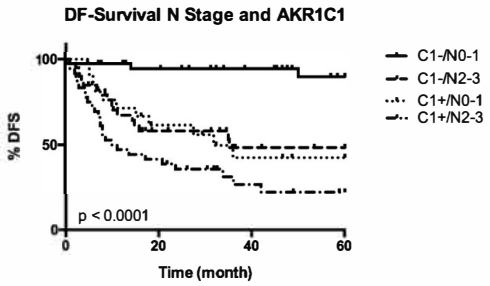

Survival of Alcohol and AKR1C1
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/Alcohol-
C1-/Alcohol+
C1+/Alcohol-
C1+/Alcohol+

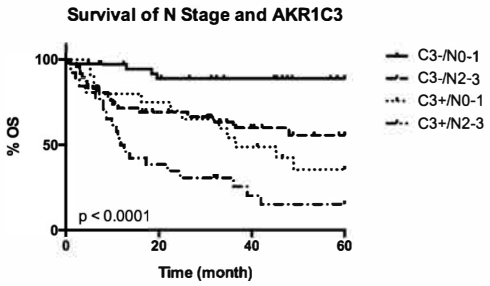

DF-Survival of Alcohol and AKR1C1
% DFS
100
50
0
p = 0.004
0
20
40
60
Time (month)
C1-/Alcohol-
C1-/Alcohol+
C1+/Alcohol-
C1+/Alcohol+

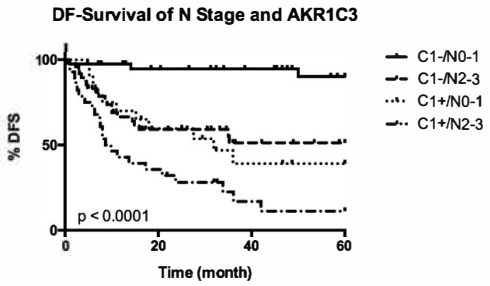

Survival of Alcohol and AKR1C3
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C3-/Alcohol-
C3-/Alcohol+
C3+/Alcohol-
C3+/Alcohol+

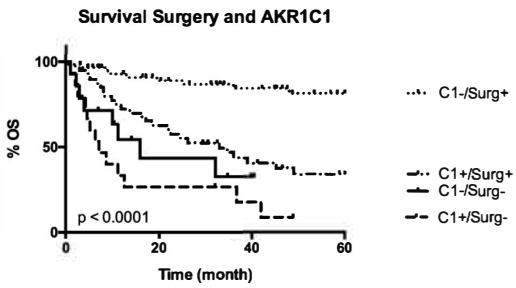

DF-Survival of Alcohol and AKR1C3
% DFS
100
50
0
p = 0.003
0
20
40
60
Time (month)
C3-/Alcohol-
C3-/Alcohol+
C3+/Alcohol-
C3+/Alcohol+

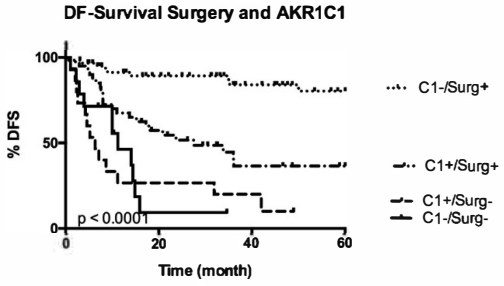

Survival N Stage and AKR1C1
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/N0-1
C1-/N2-3
C1+/N0-1
C1+/N2-3

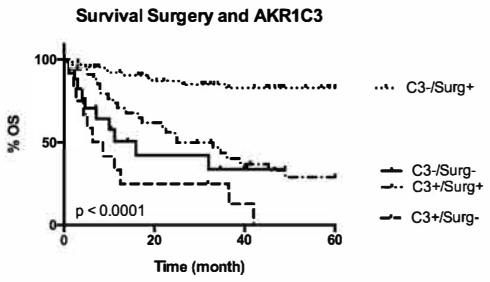

DF-Survival N Stage and AKR1C1
% DFS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/N0-1
C1-/N2-3
C1+/N0-1
C1+/N2-3

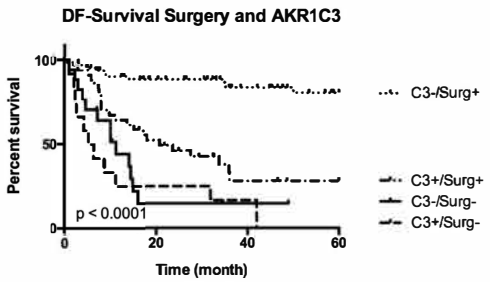

Survival of N Stage and AKR1C3
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C3-/N0-1
C3-/N2-3
C3+/N0-1
C3+/N2-3

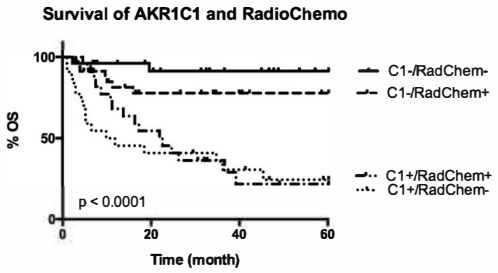

DF-Survival of N Stage and AKR1C3
% DFS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/N0-1
C1-/N2-3
C1+/N0-1
C1+/N2-3

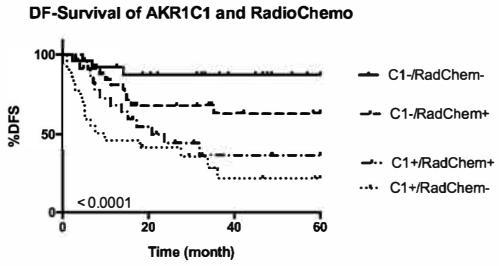

Survival Surgery and AKR1C1
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/Surg+
C1+/Surg+
C1-/Surg-
C1+/Surg-

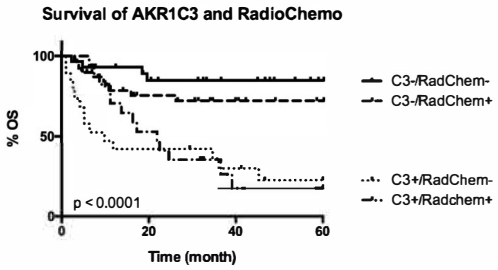

DF-Survival Surgery and AKR1C1
% DFS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C1-/Surg+
C1+/Surg+
C1+/Surg-
C1-/Surg-

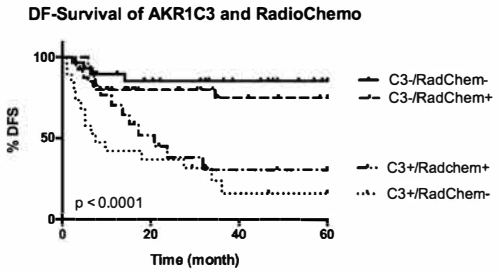

Survival Surgery and AKR1C3
% OS
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C3-/Surg+
C3-/Surg-
C3+/Surg+
C3+/Surg-

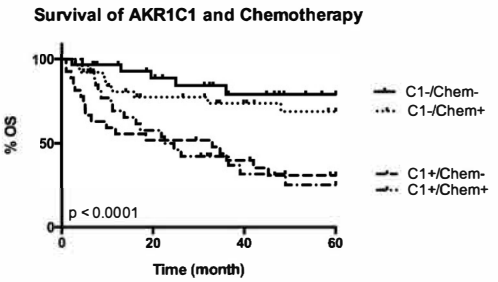

DF-Survival Surgery and AKR1C3
Percent survival
100
50
0
p < 0.0001
0
20
40
60
Time (month)
C3-/Surg+
C3+/Surg+
C3-/Surg-
C3+/Surg-

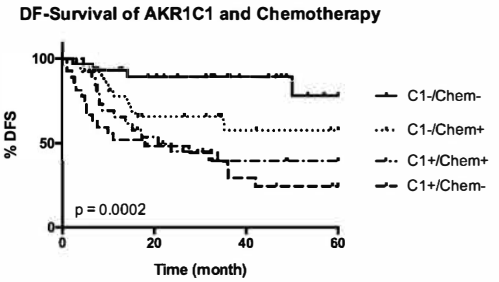

Survival of AKR1C1 and RadioChemo
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/RadChem-
C1-/RadChem+
C1+/RadChem+
C1+/RadChem-

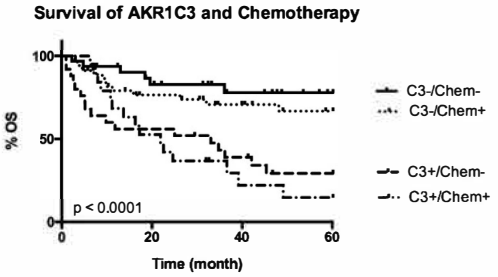

DF-Survival of AKR1C1 and RadioChemo
%DFS
100
50
0
0
20
40
60
Time (month)
< 0.0001
C1-/RadChem-
C1-/RadChem+
C1+/RadChem+
C1+/RadChem-

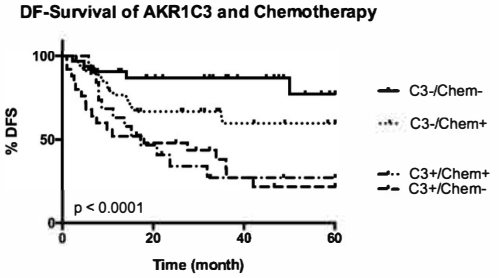

Survival of AKR1C3 and RadioChemo
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/RadChem-
C3-/RadChem+
C3+/RadChem-
C3+/Radchem+


DF-Survival of AKR1C3 and RadioChemo
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/RadChem-
C3-/RadChem+
C3+/Radchem+
C3+/RadChem-


Survival of AKR1C1 and Chemotherapy
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C1-/Chem-
C1-/Chem+
C1+/Chem-
C1+/Chem+


DF-Survival of AKR1C1 and Chemotherapy
% DFS
100
50
0
0
20
40
60
Time (month)
p = 0.0002
C1-/Chem-
C1-/Chem+
C1+/Chem+
C1+/Chem-


Survival of AKR1C3 and Chemotherapy
% OS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/Chem-
C3-/Chem+
C3+/Chem-
C3+/Chem+


DF-Survival of AKR1C3 and Chemotherapy
% DFS
100
50
0
0
20
40
60
Time (month)
p < 0.0001
C3-/Chem-
C3-/Chem+
C3+/Chem+
C3+/Chem-

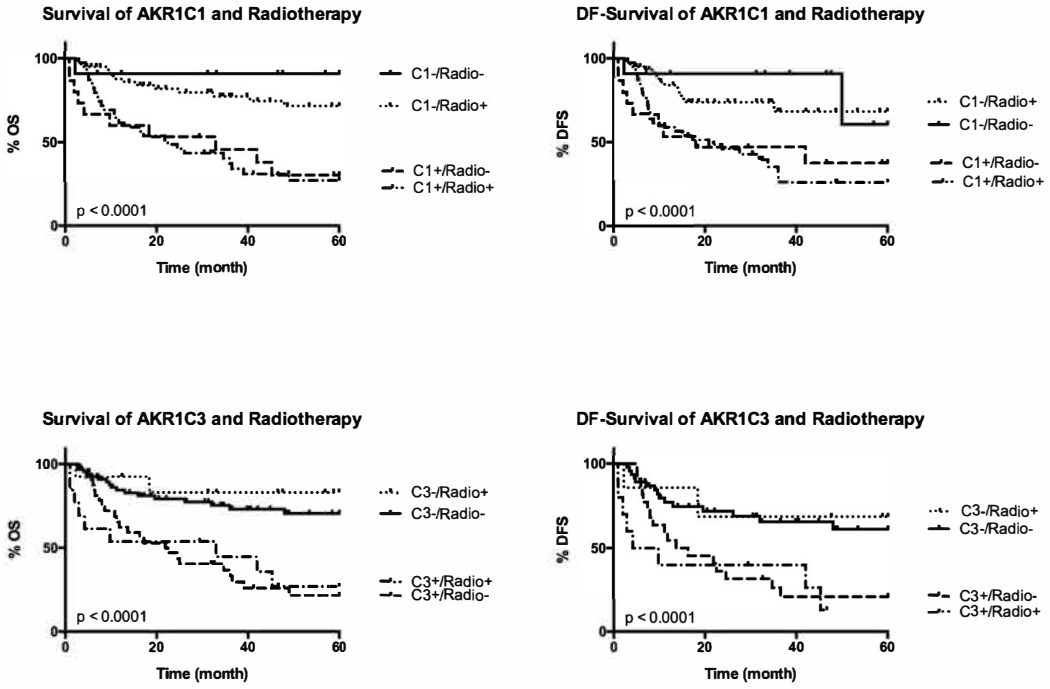


Figure S6.7 Combined univariate survival analysis for AKR1C1 and AKR1C3 expression status combined with parameters as indicated. Kaplan-Meier plot for overall survival (OS) and disease free survival (DFS) in patients with low vs. high AKR1C1 and AKR1C3 protein expression. P value was derived by log-rank/Mantel-Cox test.

Table S6.1 Summary of clinicopathological features of patients analyzed in cohort II of this study. n = Number of patients.

			HPV-Status						
	Total		HPVint		HPVint+epi		HPVepi		
Clinicopathological feature	n	%	n	%	n	%	n	%	x^2
Mean age (years)	33 (62.7)		9 (63.4)	28.1	4 (65.8)	12.5	20 (61.7)	59.4	0.733
Gender									
Male	24	72.7	6	18.2	4	12.1	14	32.4	
Female	9	27.3	3	9.1	0	0	6	18.2	0.419
T classification									
pT1 and pT2	23	69.7	6	18.2	3	9.1	14	42.4	
pT3 and pT4	10	30.3	3	9.1	1	3.0	6	18.2	0.954
N classification									
pN0	4	12.1	1	3.0	0	0	3	9.1	
pN1-2	29	87.9	8	24.2	4	12.1	17	51.5	0.396
M classification									
pM0	32	97.0	9	27.3	4	12.1	19	57.6	
pM1	1	3.0	0	0	0	0	1	3.0	0.751
AJCC classification									
I	10	30.3	4	12.1	0	0	6	18.2	
II	17	51.5	2	6.1	3	9.1	12	36.4	
III	5	15.2	3	9.1	1	3.0	1	3.0	
IV	1	3.0	0	0	0	0	1	3.0	0.221
Relapse									
Yes	12	36.4	2	6.1	2	6.1	8	24.2	
No	21	63.6	7	21.2	2	6.1	12	36.4	0.545
Death									
Yes	10	30.3	1	3.0	2	6.1	7	21.2	
No	23	69.7	8	24.2	2	6.1	13	39.4	0.285
Smoking									
Yes	17	54.8	4	12.9	2	6.5	11	35.5	
No	14	45.2	5	16.1	2	6.5	7	22.6	0.699
Alcohol									
Yes	22	71.0	6	19.4	2	6.5	14	45.2	
No	9	29.0	3	9.7	2	6.5	4	12.9	0.512
Localization									
Base of tongue	4	12.1	2	6.1	0	0	2	6.1	
Tonsil	29	87.9	7	21.2	4	12.1	18	54.5	0.473

Summary of clinicopathological features of patients analyzed in this study. n = Number of patients. Staging was performed according to AJCC/UICC 8th Edition in Oropharyngeal Squamous Cell Carcinoma. x^2: Chi-Square test for significance. For mean age, Anova is used to measure significance. Significant values are highlighted in bold.

Table S6.2 List of mRNAs found upregulated by mRNA array analysis in HPV16-positive OPSCC harboring integrated virus

Gene Symbol	Entrez GeneID	Genbank Accession	Mean Integrated	Mean Episomal and Episomal + Integrated	x-fold upregu-lation	FDR adjusted P-Value
CD8B	926	NM_172099	4,699	16,021	-3,4	2,025E-02
BCL11A	53335	NM_022893	5,765	12,377	-2,1	2,355E-02
BCL2	596	NM_000633	1,851	5,587	-3,0	1,884E-02
C3orf17	25871	NM_015412	0,774	1,553	-2,0	4,238E-03
MFSD4	148808	NM_181644	0,216	1,063	-4,9	1,299E-02
MECOM	2122	NM_004991	0,057	0,143	-2,5	5,286E-03
CD8B	926	NM_172102	0,314	0,882	-2,8	1,576E-02
CUL3	8452	NM_003590	0,133	0,283	-2,1	1,569E-02
YPEL1	29799	NM_013313	0,521	1,049	-2,0	1,973E-02
ACOT4	122970	NM_152331	0,679	1,389	-2,0	1,834E-02
NR3C2	4306	NM_000901	0,235	0,660	-2,8	1,983E-02
TBCD	6904	NM_005993	2,332	4,944	-2,1	1,077E-02
TSPAN1	10103	NM_005727	4,010	21,408	-5,3	1,867E-02

Table S6.3 List of mRNAs found downregulated by mRNA array analysis in HPV16-positive OPSCC harboring integrated virus.

This table is available at https://onlinelibrary.wiley.com/doi/10.1002/ijc.31954

Table S6.4 List of primers used for RT-qPCR.

Name	Sequence (5' à 3')
AKR1C1-for	GTAAAGCTTTAGAGGCCAC
AKR1C1-rev	ATAAGGTAGAGGTCAACATAA
AKR1C3-for	AAGCTGGGTTCCGCCATATA
AKR1C3-rev	TGCCTGCGGTTGAAGTTTGA
BCL2-for	TGTTGTTCAAACGGGATTCA
BCL2-rev	GGCTGGGCACATTTACTGTT
BCL2L10-for	GCAAATGGCTCTTCCTTGAG
BCL2L10-rev	AGCAGCACATGAAGTTGTGG
*E6*I-for*	AATGTTTCAGGACCCACA
*E6*I-rev*	GTTAATACACTCACGTCCGCAG
HPRT-for	CACTGGCAAAACAATGCAGACT
HPRT-rev	GTCTGGCTTATATCCAACACTTCGT

Table S6.5 Correlation between staining intensities of AKR1C1 and AKR1C3 immunohistochemical stainings from tumor tissue and adjacent normal epithelium.

AKR1C1

		Epithelium				
		No Staining	Weak Staining	Medium staining	Strong Staining	
Tumor	No Staining	20 (14.2%)	9 (6.4%)	0 (0%)	0 (0%)	
	Weak Staining	22 (15.6%)	22 (15.6%)	2 (1.4%)	0 (0%)	
	Medium staining	2 (1.4%)	31 (22.0%)	24 (17.0%)	0 (0%)	
	Strong Staining	1 (0.7%)	2 (1.4%)	1 (0.7%)	5 (3.5%)	$\chi 2 < 0.0001$

AKR1C3

		Epithelium				
		No Staining	Weak Staining	Medium staining	Strong Staining	
Tumor	No Staining	32 (22.7%)	11 (7.8%)	2 (1.4%)	0 (0%)	
	Weak Staining	31 (22.0%)	23 (16.3%)	3 (2.1%)	0 (0%)	
	Medium staining	2 (1.4%)	17 (12.1%)	17 (12.1%)	0 (0%)	
	Strong Staining	0 (0%)	1 (0.7%)	0 (0%)	2 (1.4%)	$\chi 2 < 0.0001$

$\chi 2$: Chi-Square test for significance.

Table S6.6 Correlation between staining intensities from normal epithelium and tumor tissue for oxidative stress related markers AKR1C3, 3-Nitrotyrosine and NRF2.

HPV-negative (n = 11)

		NRF2 Epithelium					AKR1C3 Epithelium					3-Nitrotyrosine Epithelium				
		No Staining	Weak Staining	Medium staining	Strong Staining	2	No Staining	Weak Staining	Medium staining	Strong Staining	2	No Staining	Weak Staining	Medium staining	Strong Staining	2
Tumor	No Staining	0 (0%)	0 (0%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	Weak Staining	3 (27.3%)	1 (9.1%)	0 (0%)	0 (0%)		2 (18.2%)	1 (9.1%)	0 (0%)	0 (0%)		1 (9.1%)	1 (9.1%)	0 (0%)	0 (0%)	
	Medium staining	1 (9.1%)	5 (45.5%)	0 (0%)	0 (0%)		0 (0%)	7 (63.6)	1 (9.1%)	0 (0%)		1 (9.1%)	6 (54.5%)	1 (9.1%)	0 (0%)	
	Strong Staining	0 (0%)	0 (0%)	1 (9.1%)	0 (0%)	0.005	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.037	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	0.724

HPV-positive (n = 13)

		NRF2 Epithelium					AKR1C3 Epithelium					3-Nitrotyrosine Epithelium				
		No Staining	Weak Staining	Medium staining	Strong Staining	2	No Staining	Weak Staining	Medium staining	Strong Staining	2	No Staining	Weak Staining	Medium staining	Strong Staining	2
Tumor	No Staining	0 (0%)	0 (0%)	0 (0%)	0 (0%)		0 (0%)	2 (15.4%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)	0 (0%)	0 (0%)	
	Weak Staining	1 (7.7%)	1 (7.7%)	0 (0%)	0 (0%)		1 (7.7%)	1 (7.7%)	0 (0%)	0 (0%)		1 (7.7%)	1 (7.7%)	1 (7.7%)	0 (0%)	
	Medium staining	0 (0%)	7 (53.8%)	2 (15.4%)	0 (0%)		0 (0%)	9 (69.2%)	0 (0%)	0 (0%)		6 (46.2%)	3 (23.1%)	0 (0%)	1 (7.7%)	
	Strong Staining	0 (0%)	2 (15.4%)	0 (0%)	0 (0%)	0.152	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.051	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.267

		AKR1C3 Index HPV-negative			AKR1C3 Index HPV-positive		
		Tumor lower or equal to epithelium	Tumor more than Epithelium	2	Tumor vs Epithelium Lower or Equal	Tumor more than Epithelium	2
NRF2 Index	Tumor vs Epithelium Lower or Equal	1 (9.1%)	1 (9.1%)		3 (23.1%)	0 (0%)	
	Tumor more than Epithelium	1 (9.1%)	8 (72.7%)	0.197	0 (0%)	10 (76.9%)	< 0.0001

		AKR1C3 Index HPV-negative			AKR1C3 Index HPV-positive		
		Tumor lower or equal to epithelium	Tumor more than Epithelium	2	Tumor vs Epithelium Lower or Equal	Tumor more than Epithelium	2
3-Nitrotyrosine Index	Tumor vs Epithelium Lower or Equal	2 (18.2%)	1 (9.1%)		3 (23.1%)	0 (0%)	
	Tumor more than Epithelium	0 (0%)	8 (72.7%)	0.011	0 (0%)	10 (76.9%)	< 0.0001

Table S6.7

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
Complete collection																		
All		141	77.8	58.4	-						38.1	57.7	-					
AKR1C1	≤ Epithel (-)	82	101.3	77.5	**<0.0001**		3.7 (2.1-6.4)	**<0.0001**			46.1	73.8	**<0.0001**		3.1 (1.8-5.2)	**<0.0001**		
	> Epithel (+)	59	46.4	31.6							27.9	35.1						
AKR1C3	≤ Epithel (-)	91	98.7	75.3	**<0.0001**		3.6 (2.1-6.2)	**<0.0001**			45.7	73.0	**<0.0001**		3.4 (2.0-5.7)	**<0.0001**		
	> Epithel (+)	50	42.0	27.1							25.3	29.2						
HPV	Positive	48	80.0	72.9	**0.004**		0.4 (0.2-0.8)	**0.005**			46.3	70.8	**0.006**		0.4 (0.2-0.8)	**0.007**		
	Negative	93	66.6	51.1							33.6	51.1						
T Stage	1-2	78	77.1	72.0	**0.001**		2.5 (1.5-4.3)	**0.001**			47.4	74.7	**<0.0001**		3.3 (1.9-5.7)	**<0.0001**		
	3-4	63	58.9	42.6							27.7	37.7						
N Stage	0-1	62	71.7	73.3	**<0.0001**		2.8 (1.6 - 4.9)	**<0.0001**			48.9	76.7	**<0.0001**		4.6 (1.9 - 6.5)	**<0.0001**		
	2-3	79	46.1	46.1							29.5	43.4						
Smoking	Yes	99	80.0	87.5	**0.039**		0.3 (0.1-1.0)	0.057			53.0	87.5	**0.031**		0.2 (0.1-1.0)	**0.047**		
	No	16	69.8	58.8							38.6	56.7						
Alcohol	Yes (≥2 standard drinks)	92	82.8	73.9	0.129		0.5 (0.2-1.2)	0.136			48.9	73.9	0.091		0.5 (0.2-1.1)	0.098		
	No (<2 standard drinks)	23	80.5	60.0							38.5	57.8						
Surgery	Yes	108	44.7	66.3	**< 0.0001**		0.2 (0.1 -0.4)	**<0.0001**			44.0	68.3	**< 0.0001**		0.2 (0.1 - 0.3)	**< 0.0001**		
	No	33	19.0	27.6							14.5	17.2						
Radiochemotherapy	Yes	65	51.8	78.3	0.480		1.6 (0.5 - 5.4)	0.484			49.4	78.3	0.491		0.6 (0.2 - 2.2)	0.494		
	No	62	47.9	66.7							46.9	66.7						
Chemotherapy	Yes	71	51.8	78.3	0.480		1.6 (0.5 - 5.4)	0.484			49.4	78.3	0.491		1.5 (0.5 - 5.3)	0.494		
	No	68	47.9	66.7							46.9	66.7						
Radiotherapy	Yes	106	49.0	71.0	0.302		0.4 (0.1 - 2.8)	0.323			74.2	48.0	0.778		0.8 (0.2 - 3.8)	0.779		
	No	33	56.6	88.9							77.8	52.8						
HPV and AKR1C1	HPV-/AKR1C1-	52	89.2	70.0	**< 0.0001**	**< 0.0001**	1.5 (1.1-1.8)	**0.002**	3.1 (1.7-5.8)	**<0.0001**	58.7	64.0	**< 0.0001**	**0.012**	1.4 (1.1-1.8)	**0.007**	2.1 (1.2-3.9)	**0.014**
	HPV-/AKR1C1+	39	40.0	26.3							28.3	34.2						
	HPV+/AKR1C1-	31	95.4	90.0		**0.001**			6.5 (1.8-23.5)	**0.005**	95.3	90.0		**<0.0001**			7.9 (2.2-28.3)	**0.002**
	HPV+/AKR1C1+	19	47.5	44.4							39.8	38.9						
HPV and AKR1C3	HPV-/AKR1C3-	60	84.0	65.5	**< 0.0001**	**< 0.0001**	1.4 (1.1-1.8)	**0.003**	3.1 (1.7-5.8)	**<0.0001**	59.0	63.8	**< 0.0001**	**0.012**	1.4 (1.1-1.8)	**0.004**	2.1 (1.2-3.9)	**0.014**
	HPV-/AKR1C3+	31	37.1	20.0							24.8	26.7						
	HPV+/AKR1C3-	32	98.6	93.5		**0.001**			6.5 (1.8-23.5)	**0.005**	95.9	90.3		**<0.0001**			7.9 (2.2-28.3)	**0.002**
	HPV+/AKR1C3+	18	41.7	35.3							36.3	35.3						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
Smoking and AKR1C1	Smoking+/AKR1C1-	57	100.0	75.4	**< 0.0001**	**< 0.0001**	1.6 (1.2-2.1)	**0.002**	3.4 (1.8-6.5)	**<0.0001**	74.4	70.2	**0.001**	**0.002**	1.4 (1.1-1.9)	**0.016**	2.6 (1.4-4.8)	**0.002**
	Smoking+/AKR1C1+	40	46.5	32.5							36.7	37.5						
	Smoking-/AKR1C1-	12	71.2	91.7		0.643			1.9 (0.1-33.1)	0.648	71.2	91.7		0.435			2.9 (0.2-46.0)	0.456
	Smoking-/AKR1C1+	4	57.3	75.0							47.0	75.0						
Smoking and AKR1C3	Smoking+/AKR1C3-	63	96.9	73.0	**< 0.0001**	**< 0.0001**	0.2 (0.1-1.9)	0.174	3.0 (1.5-5.7)	**< 0.0001**	72.5	68.3	**0.001**	**0.003**	1.4 (1.1-1.9)	**0.016**	2.5 (1.3-4.5)	**0.004**
	Smoking+/AKR1C3+	34	45.4	29.4							35.4	35.3						
	Smoking-/AKR1C3-	2	54.5	50.0		0.219			5.0 (0.3-82.5)	0.265	34.0	50.0		0.118			6.7 (0.4-107.9)	0.177
	Smoking-/AKR1C3-	14	72.1	92.9							72.1	93.0						
Alcohol and AKR1C1	Alcohol+/AKR1C1-	51	100.1	76.5	**< 0.0001**	**< 0.0001**	1.6 (1.2-2.2)	0.001	3.2 (1.6-6.4)	**0.001**	73.5	72.5	**0.004**	**0.003**	1.5 (1.1-2.0)	0.012	2.6 (1.4-5.1)	**0.004**
	Alcohol+/AKR1C1+	39	49.3	35.9							37.8	38.5						
	Alcohol-/AKR1C1-	18	89.6	83.3		0.104			3.5 (0.7-17.3)	0.128	84.8	77.8		0.397			2.1 (0.4-11.3)	0.407
	Alcohol-/AKR1C1+	5	44.7	40.0							43.2	60.0						
Alcohol and AKR1C3	Alcohol+/AKR1C3-	57	99.1	75.4	**< 0.0001**	**< 0.0001**	1.7 (1.2-2.2)	**0.001**	0.3 (0.2-0.6)	**0.001**	71.5	70.2	**0.003**	**0.004**	1.5 (1.1-2.1)	0.007	0.4 (0.2-0.8)	**0.005**
	Alcohol+/AKR1C3+	33	45.9	30.3							35.9	36.4						
	Alcohol-/AKR1C3-	20	86.8	80.0		0.201			2.9 (0.5-15.8)	0.222	86.7	80.0		0.082			4.1 (0.7-22.4)	0.109
	Alcohol-/AKR1C3+	3	48.4	33.3							34.7	33.3						
T Stage and AKR1C1	T1-2/AKR1C1-	50	91.9	87.5	**< 0.0001**	**< 0.0001**	2.0 (1.5-2.5)	**<0.0001**	4.7 (1.8-12.2)	**0.001**	89.1	85.4	**< 0.0001**	**0.006**	2.0 (1.6-2.5)	**< 0.0001**	**2.5 (1.3-4.8)**	**0.007**
	T1-2/AKR1C1+	28	45.8	44.4							49.6	55.6						
	T3-4/AKR1C1-	33	82.5	62.5		**0.002**			2.9 (1.4-5.8)	**0.003**	56.9	56.3		**0.005**			2.5 (1.3-4.8)	**0.007**
	T3-4/AKR1C1+	30	30.5	17.2							19.3	17.2						
T Stage and AKR1C3	T1-2/AKR1C3-	55	89.4	84.9	**< 0.0001**	**< 0.0001**	2.0 (1.6-2.5)	**<0.0001**	4.7 (1.8-12.2)	**0.001**	88.8	84.9	**< 0.0001**	**0.006**	2.0 (1.6-2.5)	**< 0.0001**	3.4 (1.3-8.6)	**0.010**
	T1-2/AKR1C3+	23	44.1	44.1							46.0	50.0						
	T3-4/AKR1C3-	37	81.2	81.2		**0.002**			2.9 (1.4-5.8)	**0.003**	56.7	55.6		**0.005**			2.5 (1.3-4.8)	**0.007**
	T3-4/AKR1C3+	26	29.3	26.9							15.9	12.0						
N Stage and AKR1C1	N0-1/AKR1C1-	39	54.9	89.7	**< 0.0001**	**< 0.0001**	2.8 (1.6 - 4.9)	**<0.0001**	2.7 (1.5 - 4.7)	**<0.0001**	89.1	92.3	**< 0.0001**	**<0.0001**	3.5 (1.9 - 6.5)	**< 0.0001**	**3.0 (1.6 - 5.7)**	**0.001**
	N0-1/AKR1C1+	21	38.4	38.1							49.6	47.6						
	N2-3/AKR1C1-	40	41.6	65.0		**0.004**			1.6 (1.2 - 2.2)	**0.004**	56.9	57.5		**0.034**			1.4 (1.0 - 1.9)	**0.037**
	N2-3/AKR1C1+	36	24.0	25.0							19.3	27.8						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
N Stage and AKR1C3	T1-2/AKR1C3-	40	54.9	90.0	**<0.0001**	**<0.0001**	2.1 (1.6 - 2.6)	**<0.0001**	2.9 (1.6 - 5.1)	**<0.0001**	92.5	56.8	**< 0.0001**	**<0.0001**	2.1 (1.6 - 2.6)	**<0.0001**	3.3 (1.7 - 6.2)	**<0.0001**
	T1-2/AKR1C3+	20	37.6	35.0							45.0	33.9						
	T3-4/AKR1C3-	48	40-4	62.5		**0.001**			1.7 (1.2 - 2.3)	**0.001**	58.3	36.7		**0.002**			1.6 (1.2 - 2.1)	**0.003**
	T3-4/AKR1C3+	28	20.8	17.9							17.9	19.0						
Surgery and AKR1C1	Surgery-/AKR1C1-	14	20.9	42.9	**< 0.0001**	0.311	0.8 (0.6 - 1.1)	0.100	1.6 (0.6 - 3.9)	0.316	12.1	21.4	**< 0.0001**	0.792	0.6 (0.5 - 0.9)	**0.004**	1.1 (0.5 - 2.6)	0.793
	Surgery-/AKR1C1+	15	15.7	13.3							14.9	13.3						
	Surgery+/AKR1C1-	61	52.9	85.2		**< 0.0001**			5.0 (2.3 - 10.8)	**<0.0001**	52.7	85.2		**<0.0001**			4.9 (2.3 - 10.6)	**< 0.0001**
	Surgery+/AKR1C1+	40	33.8	37.5							32.1	42.5						
Surgery and AKR1C3	Surgery-/AKR1C3-	17	23.3	41.2	**< 0.0001**	0.176	0.7 (0.5 - 0.9)	**0.005**	1.8 (0.8 - 4.2)	0.183	15.0	23.5	**< 0.0001**	0.432	0.7 (0.5 - 0.9)	**0.008**	1.4 (0.6 - 3.1)	0.438
	Surgery-/AKR1C3+	12	14.3	8.3							13.3	8.3						
	Surgery+/AKR1C3-	67	52.1	83.6		**< 0.0001**			5.0 (2.4 - 10.4)	**<0.0001**	52.5	85.1		**<0.0001**			5.8 (2.7 - 12.4)	**< 0.0001**
	Surgery+/AKR1C3+	34	32.0	32.4							28-aug	35.3						
Radiochemo and AKR1C1	RadioChem-/AKR1C1-	26	121.2	92.3	**< 0.0001**	**< 0.0001**	1.5 (1.1-2.0)	**0.008**	13.5 (3.1-58.9)	**0.001**	68.8	88.5	**<0.0001**	**<0.0001**	1.3 (1.0-1.7)	0.071	9.0 (2.6-31.2)	**< 0.0001**
	RadioChem-/AKR1C1+	22	39.4	27.3							24.1	27.3						
	RadioChem+/AKR1C1-	35	83.6	80.0		**< 0.0001**			4.3 (1.8-10.5)	**0.001**	71.1	68.6		0.088			2.0 (0.9-4.5)	0.094
	RadioChem+/AKR1C1+	22	32.3	22.7							39.2	40.9						
Radiochemo and AKR1C3	RadioChem-/AKR1C3-	29	113.4	86.2	**< 0.0001**	**< 0.0001**	0.1 (0.1-0.4)	**0.001**	7.6 (2.5-23.3)	**<0.0001**	67.3	86.2	**< 0.0001**	**<0.0001**	1.7 (0.8-3.7)	**0.004**	8.4 (2.8-25.5)	**< 0.0001**
	RadioChem-/AKR1C3+	19	37.6	26.3							21.1	21.1						
	RadioChem+/AKR1C3-	40	78.6	75.0		**0.001**			3.5 (1.5-7.9)	**0.003**	70.3	67.5		0.074			2.1 (0.9-4.6)	0.080
	RadioChem+/AKR1C3+	17	30.5	17.6							35.9	35.3						
Chemo and AKR1C1	Chem-/AKR1C1-	30	51.8	83.3	**< 0.0001**	**< 0.0001**	1.4 (1.0 - 1.8)	**0.023**	5.1 (1.9 - 13.7)	**0.001**	53.3	86.7	**<0.0001**	**<0.0001**	1.3 (1.0 - 1.7)	0.035	7.2 (2.5 - 21.3)	**< 0.0001**
	Chem-/AKR1C1+	27	29.4	33.3							26.1	29.6						
	Chem+/AKR1C1-	39	46.8	74.4		**< 0.0001**			3.0 (1.4 - 6.5)	**0.006**	40.9	64.1		0.133			1.7 (0.8 - 6.6)	0.139
	Chem+/AKR1C1+	26	30.3	30.8							31.7	42.3						
Chemo and AKR1C3	Chem-/AKR1C3-	32	50.6	81.3	**< 0.0001**	**< 0.0001**	1.4 (1.1 - 1.8)	**0.019**	4.5 (1.8 - 11.5)	**0.001**	52.1	84.4	**< 0.0001**	**< 0.0001**	1.4 (1.1 - 1.8)	**0.020**	6.4 (2.4 - 17.3)	**< 0.0001**
	Chem-/AKR1C3+	25	29.4	32.0							25.5	28.0						
	Chem+/AKR1C3-	46	45.5	71.7		**0.001**			3.2 (1.5 - 6.9)	**0.002**	41.5	65.2		**0.025**			2.3 (1.1 - 4.8)	**0.029**
	Chem+/AKR1C3+	19	26.9	21.1							26.8	31.6						
Radio and AKR1C1	Radio-/AKR1C1-	11	54.8	90.9	**< 0.0001**	**0.011**	1.6 (1.1 - 2.3)	**0.012**	9.1 (1.2 - 71.1)	**0.011**	51.7	81.8	**<0.0001**	**0.053**	1.5 (1.1 - 2.2)	**0.024**	4.1 (0.9 - 18.8)	**0.074**
	Radio-/AKR1C1+	15	30.1	33.3							29.6	40.0						
	Radio+/AKR1C1-	61	48.6	77.0		**< 0.0001**			3.7 (1.9 - 7.0)	**<0.0001**	45.8	73.8		**<0.0001**			3.1 (1.7 - 5.9)	**< 0.0001**
	Radio+/AKR1C1+	39	29.2	30.8							27.4	33.3						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
Radio and AKR1C3	Radio-/AKR1C3-	13	51.7	84.6	**< 0.0001**	**0.010**	1.4 (1.1 - 1.8)	**0.010**	5.9 (1.3 - 27.2)	**0.024**	53.3	84.6	**< 0.0001**	0.008	1.3 (1.0 - 1.7)	**0.020**	6.2 (1.3 - 28.7)	0.020
	Radio-/AKR1C3+	13	28.6	30.8							25.2	30.8						
	Radio+/AKR1C3-	68	47.4	75.0		**< 0.0001**			3.7 (1.9 - 6.8)	**<0.0001**	44.9	72.1		**<0.0001**			3.2 (1.7 - 5.9)	**< 0.0001**
	Radio+/AKR1C3+	32	27.7	25.0							24.8	28.1						
HPV-positive																		
T stage	HPV+/T1-2	32	50.9	81.3	0.114		0.9 (0.8 - 7.2)	0.115			86.7	81.3	**0.039**		1.1 (1.0 - 8.4)	**0.050**		
	HPV+/T3-4	16	45.0	56.3							57.1	50.0						
N stage	HPV+/N0-1	25	56.7	76.2	**0.050**		5.3 (1.4 - 19.3)	**0.012**			53.9	84.0	**0.040**		5.8 (1.9 - 23.6)	0.058		
	HPV+/N2	23	39.9	50.0							40.1	60.9						
AKR1C1	HPV+/AKR1C1-	30	95.4	90.0	**0.001**		6.5 (1.8-23.5)	**0.005**			95.3	90.0	**< 0.0001**		7.9 (2.2-28.3)	**0.002**		
	HPV+/AKR1C1+	18	47.5	44.4							39.8	38.9						
AKR1C3	HPV+/AKR1C3-	31	98.6	93.5	**0.001**		6.5 (1.8-23.5)	**0.005**			95.9	90.3	**< 0.0001**		7.9 (2.2-28.3)	**0.002**		
	HPV+/AKR1C3+	17	41.7	35.3							36.3	35.3						
Surgery	Yes	39	50.3	74.4	**0.007**		0.2 (0.0 - 0.7)	**0.016**			48.6	74.4	**0.021**		0.2 (0.1 - 0.9)	**0.035**		
	No	5	20.1	40.0							18.0	40.0						
Radiochemotherapiy	Yes	44	32.2	45.2	0.218		1.5 (0.8 - 2.7)	0.221			29.3	42.9	0.196		1.5 (0.8 - 2.8)	0.199		
	No	42	39.5	59.1							38.0	59.1						
Chemotherapy	Yes	42	32.2	45.2	0.318		0.7 (0.4 - 1.4)	0.321			33.2	50.7	0.613		1.2 (0.6 - 2.5)	0.614		
	No	42	38.4	57.1							30.9	47.1						
Radiotherapy	Yes	69	36.3	53.6	0.249		1.5 (0.7 - 3.1)	0.253			38.2	58.0	0.931		1.0 (0.5 - 2.0)	0.931		
	No	17	30-sep	41.2							38.7	57.7						
T stage and AKR1C1	HPV+/T1-2/AKR1C1-	23	96.5	91.3	**0.009**	**0.026**	1.8 (1.1 - 2.8)	**0.013**	5.5 (1.0 - 20.2)	**0.049**	96.4	91.3	**0.001**	**0.022**	2.0 (1.3 - 3.2)	**0.002**	5.6 (1.0 - 30.6)	**0.047**
	HPV+/T1-2/AKR1C1+	9	51.8	55.6							48.8	55.6						
	HPV+/T3-4/AKR1C1-	7	89.1	85.7		0.058			6.1 (0.7 - 51.9)	0.095	89.1	85.7		**0.011**			8.1 (1.0 - 66.7)	0-051
	HPV+/T3-4/AKR1C1+	9	36.0	33.3							25.4	22.2						
T stage and AKR1C3	HPV+/T1-2/AKR1C3-	23	100.5	95.7	**< 0.0001**	**0.001**	2.0 (1.2 - 3.3)	**0.005**	14.5 (1.7 - 124.0)	**0.015**	96.7	91.3	**< 0.0001**		2.2 (1.3 - 3.5)	**0.002**	5.9 (1.1 - 32.2)	**0.041**
	HPV+/T1-2/AKR1C3+	9	45.4	44.4							48.8	55.6						
	HPV+/T3-4/AKR1C3-	8	90.6	87.5		**0.013**			10.0 (1.1 - 88.2)	**0.038**	90.7	87.5		**0.003**			12. 8 (1.5 - 108.5)	**0.019**
	HPV+/T3-4/AKR1C3+	8	31.8	25.0							19.5	12.5						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
N stage and AKR1C1	HPV+/N0-1/AKR1C1-	19	58.1	94.7	**0.003**	0.099	2.2 (1.4 - 3.7)	**<0.0001**	2.4 (0.7 - 8.1)	0.146	59.2	94.7	**0.002**	**0.008**	2.4 (1.4 - 4.0)	**0.001**	3.0 (1.6 - 5.7)	**0.001**
	HPV+/N0- 1/AKR1C1+	6	53.2	66.7							37.8	50.0						
	HPV+/N2/AKR1C1-	12	47.6	75.0		0.135			1.7 (0.8 - 3.2)	0.151	51.0	83.3		**0.050**			1.4 (1.0 - 1.9)	0.037
	HPV+/N2/AKR1C1+	11	32.5	36.4							29.7	36.4						
N stage and AKR1C3	HPV+/N0- 1/AKR1C3-	19	54.9	100	**< 0.0001**	**< 0.0001**	2.9 (1.6 - 5.0)	**<0.0001**	2.8 (1.6 - 4.9)	**< 0.0001**	59.3	94.7	**< 0.0001**	**<0.0001**	2.6 (1.5 - 4.4)	**<0.0001**	3.3 (1.7 - 6.2)	**<0.0001**
	HPV+/N0-1/AKR1C3+	6	38.2	50.0							51.7	50.0						
	HPV+/N2/AKR1C3-	13	25.0	76.9		**0.009**			1.7 (1.2 - 2.3)	**0.001**	51.7	84.6		**0.041**			1.6 (1.2 - 2.1)	**0.004**
	HPV+/N2/AKR1C3+	10	20.1	30.0							26.5	30.0						
Smoking and AKR1C1	HPV+/AKR1C1-/Smoking -	10	60.4	90.0	**0.006**	0.750	2.6 (1.3 - 5.2)	**0.007**	1.6 (0.1 - 27.2)	0.752	60.4	90.0	**0.001**	0.529	2.8 (1.4 - 5.5)	**0.003**	2.4 (0.1 - 38.0)	0.542
	HPV+/AKR1C1+/Smoking -	4	57.3	75.0							47.0	75.0						
	HPV+/AKR1C1-/Smoking +	15	99.7	93.3		**0.003**			12.1 (1.5 - 99.1)	**0.020**	100.5	93.3		**<0.0001**			16.6 (2.0 - 134.5)	**0.009**
	HPV+/AKR1C1+/Smoking +	12	44.9	41.7							36.5	33.3						
Smoking and AKR1C3	HPV+/AKR1C3-/Smoking -	12	61.5	91.7	**0.001**	0.275	3.2 (1.5 - 7.0)	**0.003**	4.2 (0.3 - 70.7)	0.314	61.5	91.7	**0.002**	0.162	2.6 (1.4 - 5.0)	**0.003**	5.7 (0.4 - 91.9)	0.216
	HPV+/AKR1C3+/Smoking -	2	54.5	50.0							34.0	50.0						
	HPV+/AKR1C3-/Smoking +	14	n.a.	100.0		< 0.0001			114.7 (0.3-1000)	0.119	100.5	92.9		**0.001**			15.1 (1.8 . 123.5)	**0.011**
	HPV+/AKR1C3+/Smoking +	13	n.a.	38.5							38.5	38.5						
Surgery and AKR1C1	HPV+/Surgery-/AKR1C1-	2	35.3	50.0	**0.003**	0.502	1.0 (0.4 - 2.4)	0.957	2.3 (0.2 - 26.5)	0.512	25.3	50.0	**0.001**	0.502	1.4 (0.6 - 3.5)	0.457	2.3 (0.2 - 26.6)	0.512
	HPV+/Surgery-/AKR1C1+	3	15.7	33.3							12.3	33.3						
	HPV+/Surgery+/AKR1C1-	26	55.0	88.5		**0.012**			4.8 (1.2 - 18.5)	**0.024**	57.0	92.3		**<0.0001**			9.6 (2.0 - 45.3)	**0.001**
	HPV+/Surgery+/AKR1C1+	13	42.4	46.2							33.4	38.5						
Surgery and AKR1C3	HPV+/Surgery-/AKR1C3-	2	25.3	50.0	**< 0.0001**	0.502	1.5 (0.6 - 3.8)	0.433	2.3 (0.2 - 26.5)	0.512	25.3	50.0	**< 0.0001**	0.502	1.6 (0.6 - 4.3)	0.316	2.3 (0.2 - 26.5)	0.512
	HPV+/Surgery-/AKR1C3+	3	15.7	33.3							12.3	33.3						
	HPV+/Surgery+/AKR1C3-	27	56.6	92.6		**< 0.0001**			10.7 (2.3- 50.8)	**0.003**	57.2	92.6		**<0.0001**			12.7 (2.7 - 60.7)	**0.001**
	HPV+/Surgery+/AKR1C3+	12	38.0	33.3							30.3	33.3						
RadioChemo and AKR1C1	HPV+/AKR1C1-/RadioChemo-	5	n.a.	100	**0.012**	0.593	3.0 (1.3 - 7.3)	**0.012**	29.7 (0 - 1000)	0.742	n.a.	100	**0.001**	0.593	5.3 (1.1 - 25.1)	**0.038**	n.a.	n.a.
	HPV+/AKR1C1-/RadioChemo+	15	n.a.	93.3							n.a.	93.3					n.a.	
	HPV+/AKR1C1+/RadioChemo-	3	26.7	33.3		0.871			0.7 (0.1 - 5.3)	0.871	22.1	0		0.609			0.7 (0.1 - 7.9)	0.775
	HPV+/AKR1C1+/RadioChemo+	5	42.1	40.0							41.7	40.0						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
RadioChemo and AKR1C3	HPV+/AKR1C3-/RadioChemo-	5	n.a.	100	**0.012**	0.593	3.0 (1.3 - 7.3)	**0.012**	29.7 (0 - 1000)	0.742	n.a.	100	**0.001**	0.593	5.3 (1.1 - 25.1)	**0.038**	n.a.	n.a.
	HPV+/AKR1C3-/RadioChemo+	15	n.a.	93.3							n.a.	93.3					n.a.	
	HPV+/AKR1C3+/RadioChemo-	3	26.7	33.3		0.871			0.7 (0.1 - 5.3)	0.871	22.1	0		0.609			0.7 (0.1 - 7.9)	0.775
	HPV+/AKR1C3+/RadioChemo+	5	42.1	40.0							41.7	40.0						
Chemo and AKR1C1	HPV+/Chem-/AKR1C1-	9	52.1	77.8	**0.1407**	0.385	1.1 (0.6 - 2.0)	0.722	2.2 (0.4 - 13.0)	0.397	57.5	88.9	**0.044**	**0.044**	1.3 (0.7 - 2.4)	0.434	7.0 (0.8 - 62.4)	0.083
	HPV+/Chem-/AKR1C1+	6	41.9	50.0							32.9	28.6						
	HPV+/Chem+/AKR1C1-	15	56.9	93.3		**0.034**			7.5 (0.8 - 67.3)	**0.034**	56.9	93.3		**0.021**			8.6 (1.0 - 77.1)	0.054
	HPV+/Chem+/AKR1C1+	8	43.7	50.0							36.5	50.0						
Chemo and AKR1C3	HPV+/Chem-/AKR1C3-	8	56.0	87.5	**0.007**	**< 0.0001**	1.4 (0.7 - 2.7)	0.373	5.2 (0.6 - 46.2)	0.143	57.5	87.5	**0.002**	0.061	1.4 (0.7 - 2.9)	**0.009**	6.3 (0.7 - 56.5)	0.102
	HPV+/Chem-/AKR1C3+	7	39.5	42.9							34.7	42.9						
	HPV+/Chem+/AKR1C3-	17	57.2	94.1		**0.001**			13.9 (1.5 - 125.3)	**0.019**	57.2	94.1		**0.002**			14.7 (1.6 - 132.0)	0.017
	HPV+/Chem+/AKR1C3+	6	37.6	33.3							28.7	33.3						
Radio and AKR1C1	HPV+/Radio-/AKR1C1-	6	90.2	100	0.065	0.157	2.8 (1.0 - 7.5)	**0.042**	1.7 (0.1 - 27.9)	0.698	56.7	83.3	**0.003**	0.695	2.4 (0.9 - 6.2)	0.064	1.7 (0.1 - 27.9)	**0.698**
	HPV+/Radio-/AKR1C1+	3	71.4	66.7							43.7	66.7						
	HPV+/Radio+/AKR1C1-	20	54.1	85.0		**0.014**			16.1 (2.0 - 131.4)	**0.009**	57.7	95.0		**<0.0001**			16.1 (2.0 - 131.4)	0.009
	HPV+/Radio+/AKR1C1+	11	34.4	45.5							32.0	36.4						
Radio and AKR1C3	HPV+/Radio-/AKR1C3-	6	90.2	100	**0.001**	**< 0.0001**	1.5 (0.9 - 2.6)	**0.145**	1.7 (0.1 - 27.9)	0.698	57.8	95.2	**< 0.0001**	0.695	1.5 (0.9 - 2.6)	0.145	1.7 (0.1 - 27.9)	0.698
	HPV+/Radio-/AKR1C3+	3	71.4	66.7							26.9	30.0						
	HPV+/Radio+/AKR1C3-	21	56.3	90.5		0.197			22.9 (2.8 - 189.6)	**0.004**	56.7	83.3		**<0.0001**			22.9 (2.8 - 189.6)	**0.004**
	HPV+/Radio+/AKR1C3+	10	34.2	30.0							43.7	66.7						
HPV-negative																		
T stage	HPV-/T1-2	46	5.4	65.1	**0.009**		2.3 (1.2 - 4.3)	**0.009**			58.2	69.8	**< 0.0001**		3.2 (1.6 - 6.1)	**< 0.0001**		
	HPV-/T3-4	47	4.1	36.4							33.0	33.3						
N stage	HPV-/N0-1	37	5.7	65.6	**0.007**		2.6 (1.3 - 5.2)	**0.004**			73.8	78.1	**< 0.0001**		4.6 (2.0 - 10.5)	**< 0.0001**		
	HPV-/N2-3	56	4.5	42.3							25.4	37.7						
AKR1C1	HPV-/AKR1C1-	53	89.2	70.0	**< 0.0001**		1.5 (1.1 - 1.8)	**0.002**			58.7	64.0	**< 0.0001**		1.4 (1.1 - 1.8)	**0.007**		
	HPV-/AKR1C1+	40	40.0	26.3							28.3	34.2						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
AKR1C3	HPV-/AKR1C3-	61	84.0	65.5	**< 0.0001**		1.4 (1.1-1.8)	**0.003**			59.0	63.8	**< 0.0001**		1.4 (1.1-1.8)	**0.004**		
	HPV-/AKR1C3+	32	37.1	20.0							24.8	26.7						
Surgery	Yes	62	41.0	61.3	**< 0.0001**		0.3 (0.2 - 0.5)	**<0.0001**			40.7	64.5	**< 0.0001**		0.2 (0.1 - 0.4)	**< 0.0001**		
	No	24	18.2	25.0							12.5	12.5						
Radiochemotherapiy	Yes	65	39.4	56.9	0.640		1.1 (0.7 - 2.0)	0.640			36.9	55.4	0.616		1.2 (0.7 - 2.0)	0.617		
	No	59	41.5	61.0							40.2	61.0						
Chemotherapy	Yes	65	39.4	56.9	0.780		0.9 (0.5 - 1.6)	0.924			36.9	55.4	0.698		0.9 (0.5 - 1.6)	0.698		
	No	57	40.8	59.6							39.7	59.6						
Radiotherapy	Yes	100	40.4	59.0	0.825		1.1 (0.6 - 2.1)	0.825			38.2	58.0	0.931		1.0 (0.5 - 2.0)	0.931		
	No	26	39.8	57.7							38.7	57.7						
T stage and AKR1C1	HPV-/T1-2/AKR1C1-	27	82.0	84.0	**< 0.0001**	**0.018**	1.8 (1.4 - 2.5)	**<0.0001**	3.6 (1.2 - 11.4)	**0.027**	66.8	80.0	**< 0.0001**	0.152	1.9 (1.4 - 2.6)	**< 0.0001**	2.2 (0.7 - 6.8)	1.163
	HPV-/T1-2/AKR1C1+	19	82.0	38.9							40.7	55.6						
	HPV-/T3-4/AKR1C1-	27	73.1	56.0		**0.004**			2.9 (1.4 - 6.1)	**0.006**	44.0	48.0		**0.032**			2.2 (1.1 - 4.5)	**0.037**
	HPV-/T3-4/AKR1C1+	20	24.0	10.0							15.1	15.0						
T stage and AKR1C3	HPV-/T1-2/AKR1C3-	32	75.1	76.7	**< 0.0001**	0.072	1.8 (1.3 - 2.3)	**<0.0001**	2.4 (0.9 - 6.7)	0.086	67.1	80.0	**< 0.0001**	0.072	1.9 (1.4 - 2.6)	**< 0.0001**	2.6 (0.9 - 7.8)	0.084
	HPV-/T1-2/AKR1C3+	14	38.0	38.5							37.0	46.2						
	HPV-/T3-4/AKR1C3-	29	70.5	53.6		**0.005**			2.8 (1.3 - 5.9)	**0.006**	43.1	46.4		**0.028**			2.2 (1.1 - 4.5)	**0.032**
	HPV-/T3-4/AKR1C3+	18	21.6	5.9							14.0	11.8						
N stage and AKR1C1	HPV-/N0-1/AKR1C1-	22	87.8	89.5	**< 0.0001**	**0.002**	1.5 (1.0 - 2.1)	**0.034**	7.9 (1.7 - 36.9)	**0.009**	88.6	94.7	**< 0.0001**	**0.009**	2.1 (1.5 - 2.9)	**< 0.0001**	10.0 (1.2 - 84.1)	**0.034**
	HPV-/N0-1/AKR1C1+	14	44.0	30.8							41.2	53.8						
	HPV-/N2-3/AKR1C1-	32	72.2	58.6		**0.016**			2.4 (1.1 - 4.9)	**0.020**	30.0	48.3		**0.159**			1.6 (0.8 - 3.2)	**0.011**
	HPV-/N2-3/AKR1C1+	25	32.3	20.8							20.8	25.0						
N stage and AKR1C3	HPV-/N0-1/AKR1C3-	23	83.7	85.0	**< 0.0001**	**0.010**	1.4 (1.0 - 2.0)	0.052	0.4 (0.1 - 1.8)	0.215	88.9	95.0	**< 0.0001**	**0.005**	2.5 (1.7 - 3.6)	**< 0.0001**	11.1 (1.3 - 92.7)	**0.026**
	HPV-/N0-1/AKR1C3+	13	46.1	33.3							40.0	50.0						
	HPV-/N2-3/AKR1C3-	39	69.9	55.6		**0.003**			1.2 (0.6 - 2.4)	0.650	31.5	50.0		**0.013**			2.4 (1.2 - 4.7)	**0.016**
	HPV-/N2-3/AKR1C3+	18	21.8	11.8							14.6	11.8						
Smoking and AKR1C1	HPV-/AKR1C1-/Smoking -	2	n.a.	100	**0.011**	n.a.	2.7 (1.4 - 5.1)	**0.004**	n.a.	n.a.	n.a.	100	0.090	n.a.	2.0 (1.1 - 3.8)	**0.030**	n.a.	n.a.
	HPV-/AKR1C1+/Smoking -	2	n.a.	100					n.a.		n.a.	100					n.a.	
	HPV-/AKR1C1-/Smoking +	43	90.0	69.0		**0.003**			2.7 (1.4 - 5.5)	**0.005**	58.2	61.9		0.105			1.7 (0.9 - 3.5)	0.109
	HPV-/AKR1C1+/Smoking +	27	41.5	28.6							32.3	39.3						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)									Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
Smoking and AKR1C3	HPV-/AKR1C3-/Smoking -	2	n.a.	100	**0.016**	n.a.	2.5 (1.3 - 4.9)	**0.006**	n.a.	n.a.	n.a.	100	0.103	n.a.	2.0 (1.1 - 3.8)	**0.033**	n.a.	n.a.
	HPV-/AKR1C3+/Smoking -	2	n.a.	100					n.a.		n.a.	100					n.a.	
	HPV-/AKR1C3-/Smoking +	49	85.7	63.5		**0.008**			2.4 (1.2 - 4.8)	**0.011**	57.9	61.2		0.077			1.8 (0.9 - 3.7)	0.082
	HPV-/AKR1C3+/Smoking +	21	40.2	23.8							29.9	33.3						
Surgery and AKR1C1	HPV-/Surgery-/AKR1C1-	12	20.3	41.7	**< 0.0001**	0.382	0.8 (0.6 - 1.1)	0.129	1.5 (0.6 - 4.1)	0.387	9.3	16.7	**< 0.0001**	0.837	0.8 (0.6 - 1.1)	0.129	1.6 (0.6 - 4.1)	0.387
	HPV-/Surgery-/AKR1C1+	12	14.8	8.3							14.4	8.3						
	HPV-/Surgery+/AKR1C1-	35	51.2	82.9		**< 0.0001**			4.7 (1.9 - 11.8)	**0.001**	48.9	80.0		**0.005**			4.7 (1.8 - 11.8)	**0.001**
	HPV-/Surgery+/AKR1C1+	27	29.9	33.3							31.0	44.4						
Surgery and AKR1C3	HPV-/Surgery-/AKR1C3 -	15	22.5	40.0	**< 0.0001**	0.187	0.8 (0.6 - 1.0)	0.098	1.9 (0.7 - 4.7)	0.195	12.2	20.0	**< 0.0001**	0.600	0.8 (0.6 - 1.0)	0.089	1.9 (0.7 - 4.7)	0.195
	HPV-/Surgery-/AKR1C3+	9	13.0	0.0							12.5	0.0						
	HPV-/Surgery+/AKR1C3 -	40	48.7	77.5		**0.001**			3.7 (1.6 - 8.4)	**0.002**	48.8	80.0		**0.001**			3.7 (1.6 - 8.4)	**0.002**
	HPV-/Surgery+/AKR1C3+	22	29.1	31.8							27.8	36.4						
RadioChemo and AKR1C1	HPV-/AKR1C1-/RadioChemo-	21	118.7	90.5	**< 0.0001**	0.077	1.9 (1.4 - 2.6)	**<0.0001**	3.9 (0.8 - 19.2)	0.100	66.7	85.7	**0.008**	**0.014**	1.4 (1.1 - 1.9)	**0.013**	4.4 (1.2 - 16.2)	**0.013**
	HPV-/AKR1C1-/RadioChemo+	20	67.0	70.0							43.1	50.0						
	HPV-/AKR1C1+/RadioChemo-	19	38.4	26.3		0.845			0.9 (0.4 - 2.0)	0.846	25.3	31.6		0.324			0.7 (0.3 - 1.5)	0.328
	HPV-/AKR1C1+/RadioChemo+	17	29.5	17.6							26.4	41.2						
RadioChemo and AKR1C3	HPV-/AKR1C3-/RadioChemo-	24	109.7	83.3	**< 0.0001**	0.091	1.8 (1.3 - 2.4)	**<0.0001**	2.7 (0.8 - 8.7)	0.100	65.1	83.3	**0.002**	**0.021**	1.5 (1.2 - 2.1)	**0.004**	3.5 (1.1 - 10.9)	**0.031**
	HPV-/AKR1C3-/RadioChemo+	25	61.5	64.0							46.3	52.0						
	HPV-/AKR1C3+/RadioChemo-	16	26.3	25.0		0.984			1.0 (0.4 - 2.3)	0.984	21.5	25.0		0.347			0.7 (0.3 - 1.6)	0.350
	HPV-/AKR1C3+/RadioChemo+	12	25.7	8.3							24.3	33.3						

Table S6.7 (continued)

			Overall Survival (OS) (univariate)								Disease-free Survival (DFS) (univariate)							
Samples analyzed / Parameters	**Group**	**No.**	**Median OS (month)**	**5-year OS (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**	**Median DFS (month)**	**5-year DSF (%)**	**p-Value * total**	**p-Value * subgroup comparison**	**Hazard ratios (HR) (95% CI)**	**p-Value ** total**	**Hazard ratios (HR) (95% CI) subgroup comparison**	**p-Value ** subgroup comparison**
Chemo and AKR1C1	HPV-/Chem-/AKR1C1-	21	51.8	87.5	**0.001**	**0.001**	1.5 (1.1 - 1.9)	**0.012**	**6.6 (1.9 - 23.0)**	**< 0.0001**	52.0	87.7	**0.001**	**<0.0001**	1.4 (1.1 - 1.8)	**0.031**	6.8 (2.0 - 23.6)	**0.002**
	HPV-/Chem-/AKR1C1+	21	29.4	28.6							24.1	28.6						
	HPV-/Chem+/AKR1C1-	24	46.8	62.5		0.054			2.3 (1.0 - 5.3)	0.060	39.9	45.8		0.828			1.1 (0.5 - 2.5)	0.053
	HPV-/Chem+/AKR1C1+	18	30.3	22.2							25.4	38.9						
Chemo and AKR1C3	HPV-/Chem-/AKR1C3-	24	48.9	79.2	**0.009**	**0.002**	1.4 (1.1 - 1.9)	**0.015**	4.6 (1.6 - 12.9)	**0.004**	50.7	83.3	**0.002**	**<0.0001**	1.4 (1.1 - 1.9)	**0.019**	6.5 (2.1 - 19.8)	**0.001**
	HPV-/Chem-/AKR1C3+	18	25.8	27.8							21.6	22.2						
	HPV-/Chem+/AKR1C3-	29	38.1	58.6		**0.050**			2.3 (1.0 - 5.2)	**0.056**	31.3	48.3		0.512			1.3 (0.6 - 3.1)	0.514
	HPV-/Chem+/AKR1C3+	13	22.0	15.4							23.1	30.8						
Radio and AKR1C1	HPV-/Radio-/AKR1C1-	5	48.5	80.0	**0.001**	0.121	1.3 (0.9 - 2.0)	0.179	3.7 (0.5 - 29.4)	0.221	38.6	80.0	0.056	**0.190**	1.3 (0.9 - 1.9)	0.246	3.7 (0.5 - 29.4)	0.221
	HPV-/Radio-/AKR1C1+	12	24.7	25.0							25.4	33.3						
	HPV-/Radio+/AKR1C1-	41	45.6	73.2		**0.001**			2.2 (1.1 - 4.3)	**0.026**	39.1	63.4		**0.022**			2.2 (1.1 - 4.3)	0.026
	HPV-/Radio+/AKR1C1+	28	24.8	25.0							25.3	32.1						
Radio and AKR1C3	HPV-/Radio-/AKR1C3-	7	44.6	71.4	**0.002**	0.062	1.5 (1.1 - 1.9)	**0.007**	3.9 (0.8 - 18.7)	**0.084**	50.7	85.7	**0.006**	**0.026**	1.3 (1.0 - 1.7)	**0.050**	7.5 (0.9 - 60.1)	0.058
	HPV-/Radio-/AKR1C3+	10	20.0	20.0							17.2	20.0						
	HPV-/Radio+/AKR1C3-	47	43.1	68.8		**0.009**			2.8 (1.4 - 5.5)	**0.004**	38.3	61.7		**0.028**			2.1 (1.1 - 4.1)	0.028

Table S6.8 List of integration sites for tumors used in mRNA array analysis. These data were already published in Olthof et al. 2014.[3]

No.	Sex	Integation status (E = Episomal, I = Integrated)	Integration Locus
TU 1	M	I	LHFPL3 7q22.2
TU 2	F	I	FANCC 9q22.3
TU 3	M	I	SGSM1 22q11.23
TU 4	M	I	HDAC2 6q21, TRAF 3 14q32.32
TU 5	M	I	Intergenic 13q22
TU 6	M	I	Intergenic 3q27
TU 7	M	I	SYNPO2 4q26
TU 8	M	I	Intergenic 8p11.1
TU 9	M	I	C20orf26 20p11.23
TU 10	M	I	LHFPL3 7q22.2
TU 11	F	E	
TU 12	M	E	
TU 13	M	I + E	Intergenic 17q21.2
TU 14	M	E	
TU 15	M	E	
TU 16	F	E	
TU 17	M	E	
TU 18	F	E	
TU 19	F	E	
TU 20	M	I + E	Intergeniq 15q15
TU 21	M	I + E	Intergenic 8q24.21
TU 22	F	E	
TU 23	M	E	
TU 24	F	E	
TU 25	M	E	
TU 26	M	E + I	ZMAT4 8p11.21
TU 27	M	E	
TU 28	F	E	
TU 29	M	E	
TU 30	F	E	
TU 31	M	E	
TU 32	M	E	
TU 33	M	E	

Chapter 7

General discussion

General discussion

Because knowledge of HPV as a risk factor for developing OPSCC is a prerequisite for early detection and treatment of the disease, the first part of the thesis focusses on the question how this knowledge is among the general population and healthcare professionals in the Netherlands. After all, this is the basis for prevention and early referral.
The second part addresses the urgent need to improve the treatment for HNSCC, which is caused by the fact that the 5-year survival rate of HNSCCs is still around 40-50%. Could PI3K inhibitors, CDK4/6 inhibitors and/or the antiviral agent Cidofovir play a role in the treatment of HPV positive as well as HPV negative HNSCCs?
The third part focusses on the subgroup of HPV positive patients with a less favorable prognosis and a greater risk of recurrence or developing a second primary tumor than expected.[1] while in general HPV positive tumors have a far more favorable prognosis than HPV negative tumors. It is now clear that the presence of HPV in tumor cells does not predict the outcome in the individual patient. The use of HPV as a biomarker for dose de-escalation or changing of treatment modalities has therefore not been successful, yet. How HPV integration affects its host cell and whether HPV integration contributes to the prognosis in HNSCC is the subject of this part of the thesis.

1. Awareness of HPV among the general population and health care professionals in the Netherlands

While the incidence of tobacco related cancer has declined in the past two decades, there is an increase in HPV associated OPSCCs.[2,3] The incidence of OPSCCs in men overtook that of cervical cancer in the United Kingdom in 2016[4], following a similar trend observed in the USA in 2012.[5] In the Netherlands, all women between the age of 30 and 60 are invited to be screened for cervical cancer every five years. Thanks to this screening program, (pre)cancerous lesions may be detected. Currently, no effective OPSCC screening program exists because OPSCC precursor lesions are seldomly identified. Analysis of HPV detection in oral and oropharyngeal brushes has been described, however, HPV DNA detected in the oral cavity and oropharynx is unreliable to predict the presence of OPSCC.[6,7]
Since vaccination against HPV for young women became available (protecting against at least HPV types 16 and 18), the awareness of HPV as a sexually transmitted disease and as causative agent of cervical cancer, has dramatically increased.[8,9] The HPV vaccine not only protects against the development of cervical cancer, but also against other

anogenital cancers, as well as oropharyngeal cancer.[10] Therefore, it is important that HPV vaccination in men also receives sufficient attention. In order to maximize the potential benefits of HPV vaccination, it is necessary to get the vaccination coverage as high as possible for women as well as men.

Besides the importance of increasing the HPV vaccination rates, it is also important that healthcare professionals have the right knowledge about HPV and its association with oropharyngeal cancer and the clinical aspects of this disease. Correct knowledge of healthcare professionals can contribute to early diagnosis and treatment in these patients.
Until now it was unclear what the knowledge is about the role of HPV in oropharyngeal tumors under the Dutch population and general practitioners.

Chapter 2 shows the results of the study examining the awareness on HPV associated OPSCC among the Dutch population. 30.6% of the participants had heard of HPV and only 29.9% of these participants knew about the association between HPV and oropharyngeal cancer. This frequency is slightly lower in comparison with earlier studies, for example the study of Williams et al.[9] in which 36% of the respondents reported to know that HPV is a causative factor for oropharyngeal cancer. However, more than 75% of the participants in this study were aged between 18 and 35, while in our study only 17% of the respondents were aged 18-29 years and 56% aged 50-65 years. In a recent study by Lechner et al, 38.7% of the respondents knew of the association between HPV and OPSCC and the age range of the participants was comparable with that in our study.[11]
There are some limitations of our study. All Internet-based surveys incur the potential for bias by excluding participants who lack Internet connection. There is also potential for bias because of the selection of people who want to participate in a panel.
However, the results of this survey indicate that the public awareness of HPV and the association with oropharyngeal cancer is lacking. The awareness about HPV, the HPV vaccine and the link of HPV with OPSCC was greater among women and suggests that this knowledge is primarily due to awareness of the role of HPV in cervical cancer. Since the incidence of HPV related OPSCCs is 3 to 6 times higher in men than in women and the incidence of HPV related OPSCCs exceeds the incidence of HPV related cervical cancer in higher income countries[12,13], greater awareness of the role of HPV infection in OPSCCs is necessary to improve vaccine uptake, in women but especially also in men.

The awareness of the association between HPV and OPSCCs is much higher under the general practitioners (72%), but more than a quarter of the general practitioners in The

Netherlands is unaware of HPV as a causative factor for OPSCC. The awareness rate for general practitioners in this study is comparable to the awareness reported for general practitioners in the UK (74%) and Poland (80%).[14,15] In contrast, the awareness among general practitioners was lower in Jordan (43.3%), Germany (54%) and Italy (38%).[16-18] The results in **chapter 3** show that there was limited awareness among the general practitioners regarding gender, age and prognosis of patients with HPV associated OPSCCs. Only 35.5% of the participants were aware that HPV associated OPSCC patients are more often male, and just over half of the participants knew that these patients are generally younger of age. The early mentioned UK study also noticed this knowledge gap, describing that 41.5% of general practitioners identified HPV associated OPSCCs as being more common in men, and 58.8% correctly reported the association with younger age.[14]
There are some limitations in our study presented in chapter 3. To minimize response bias, general practitioners were offered the choice to complete the questionnaire via an online link or on paper. The response rate of this study was relatively low and there was no information on non-responders. Any (non) response bias that may have affected the interpretation of the results of the study could therefore not be tested (see the discussion in chapter 3).

The results of our studies support that 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.

Different interventions to increase the awareness of HPV are available and have been studied. There is an association between HPV vaccination acceptance and individual knowledge, attitudes and beliefs. Therefore many studies (mostly done in the USA) have focused on written informational handouts targeted toward educated populations, thereby not reaching populations outside the university setting.[19,20] It is known from previous studies that the HPV vaccination coverage in the Netherlands is lower among adolescents with parents with lower socio-economic status.[21,22] The literature shows that interventions such as the use of reminders, a no-show policy, tailormade information, giving feedback on the vaccination coverage to professionals and making it easier to get vaccinations, could potentially increase the HPV vaccination coverage by 10-20%.[23] Another intervention could be the start of a collaboration with for example cancer institutes and health care professionals to create a positive message among vaccination and the protection against cancer. These kind of interventions resulted in

Ireland to a vaccination coverage increase of 50.0% in 2016-2017 to 61.7% in 2017-2018.[24]

Raising awareness of HPV could also be achieved by targeting the public through the Internet, particularly through social media. Despite the growing body of literature examining social media in health contexts, limited insight has been provided into how the utility of social media may vary depending on the particular public health objective governing an intervention.[25] In the study of Taleb et al, conducted during the COVID-pandemic, social media users had a significantly higher awareness score of COVID-19 infection than that of the non-users. They concluded that social media remain a promising tool that can be used for raising public awareness.[26] As much as social media platforms seem to be successful in targeting a large audience, shared information may include also rumors and misinformation.[26,27] So to what extent social media could play a role in increasing awareness about HPV and OPSCC remains unclear.

It is obligate that health care professionals have sufficient knowledge about HPV and the role of HPV in the development of OPSCCs. If patients have questions about HPV and the vaccine, the health care professional must be able to answer these questions. This is not only the case for general practitioners, but also for a dentist for example. Recently, Poelman et al. studied the knowledge of Dutch dentists regarding HPV associated cancer of the oropharynx. 67% of 607 dentists were aware of the link between HPV and oropharyngeal cancer. More female dentists were aware of this relationship as well as the availability of an HPV vaccine. Many respondents indicated that they would like to have access to more professional literature on this subject and to have the opportunity to follow further training.[28]
There is a relatively low exposure of dentists as well as general practitioners in the Netherlands to HPV associated oropharyngeal cancer. For example, a general practitioner in the Netherlands observes on average one head and neck cancer patient every four years. Therefore, it is important that the knowledge about HPV and its association with oropharyngeal tumors is kept at a sufficient level in e.g. education courses for health care professionals.

The results of the study in Chapter 2 have already been used as information tool for the national 'Make Sense Campaign', which is a yearly initiative from the Dutch Working Group on Head and Neck Tumors (NWHTT), in order to create more awareness about head and neck cancer among the Dutch population and in this specific case about HPV oropharyngeal cancer. The results of the study in Chapter 3 have also been shared with the Dutch journal for general practitioners 'Huisarts & Wetenschap' and the general

practitioners who participated in this study received a fact sheet with information about HPV and the role of HPV in oropharyngeal cancer.

2. Treatment of HNSCC patients

The mainstay of treatment for locoregionally advanced HNSCC is either surgery followed by adjuvant radiation therapy or definitive concurrent chemoradiation with cisplatin. The heterogeneous nature of HNSCCs at the molecular level and a majority of mutations occurring particularly in tumor suppressor genes has hindered the application of targeted therapeutics to this group of tumors.[29,30] Cetuximab, a monoclonal antibody directed against Epidermal Growth Factor Receptor (EGFR) inhibiting downstream signaling, is a possible alternative to chemotherapy in patients unfit for cisplatin. For patients with recurrent or metastatic HNSCC novel immunotherapies (Nivolumab or Pembroluzimab) are an option[31], although effective in a relatively small subset of patients.
There are different pathways playing a role in the development of HPV positive and -negative HNSCCs. The most common abnormalities in HNSCCs according to the analysis of The Cancer Genome Atlas (TCGA) are involved in the cell cycle, survival, and oxidative stress response.[32]

2.1 CDK4/6 inhibitors

The most frequently affected pathway in HNSCCs is the Cyclin D-Cdk4/6-pRb pathway. Cyclin D-cyclin-dependent kinase 4/6 phosphorylates and inactivates the tumor suppressor retinoblastoma (Rb1), leading to release and activation of E2F transcription factors, necessary for G_1-S phase cell-cycle progression. In HPV positive HNSCC, the viral oncoprotein E7 drives unrestrained proliferation by promoting Rb1 degradation.[33] In HPV negative HNSCC, Rb1 inactivation occurs through hyperactivation of the Rb1 inhibitory complex CDK4/6-Cyclin D. *CCND1* (gene encoding cyclin D1, the regulatory subunit of the complex) is regularly amplified and/or the CDK4/6 inhibitor p16 (encoded by the gene *CCND2A*) is inactivated in nearly all of these cancers and prevents phosphorylation of Rb1.[34,35] Therefore, one of the hypothesis in this thesis was that CDK4/6 inhibitors are effective in HPV negative HNSCC cell lines and ineffective in HPV positive cell lines.
In **Chapter 4** HPV negative and HPV positive HNSCC cell lines were treated with two CDK4/6 inhibitors (palbociclib and ribociclib). Treatment resulted in cell growth inhibition and G_1-phase arrest in the HPV negative cell lines but not in the HPV positive

cell lines. These findings confirmed our hypothesis and are relevant for translation of these results to the clinic.

Especially in the HPV negative tumors, some studies analysed a number of biomarkers for a more optimal prediction of clinical response to CDK4/6 inhibitors. Huang et al. reported a proteogenomic study on 108 HPV negative HNSCCs and found that the Rb1 phosphorylation levels are an effective and necessary indicator of CDK4/6 dependent cell cycle activity, which cannot be accurately predicted using genomic or transcriptomic markers.[36] They also analyzed data from HPV negative HNSCC patient-derived xenograft models treated with a CDK4/6 inhibitor (abemaciclib) and found that cell lines with higher levels of Rb1 were more sensitive to treatment with CDK4/6 inhibitors.[36,37] The results of these observations support their hypothesis that phospho- or total Rb1 may serve as markers for CDK4/6 inhibitors in HPV negative HNSCCs.

There are clinical trials ongoing to access the efficacy of CDK inhibitors in HNSCC. Recently, a multicenter phase II trial has been completed and published. Palbociclib was given in combination with carboplatin in 18 patients with unresectable recurrent or metastatic HNSCCs (8 HPV negative, 4 HPV positive, 6 HPV status unknown). This combination didn't improve outcome and was associated with treatment related toxicity.[38] An earlier multicenter phase II study, involving 62 platinum-resistant or cetuximab-resistant HPV negative HNSCC patients, were given palbociclib and cetuximab. Objective response was 39% in platinum-resistant and 19% in cetuximab-resistant tumors and thus similar to or even higher than reported for PD-1 inhibitors and also higher than expected in similar patients treated with single-agent cetuximab.[39] Additional clinical trials investigating CDK inhibitors to treat HNSCC are ongoing and the results are awaited. In the UPSTREAM study of the EORTC, a multicenter pilot study proposing a therapeutic strategy based on biomarkers in patients with recurrent/metastatic HNSCC, palbociclib is indicated in patients with *CCND1* amplified and p16 negative tumors. Patients are still being recruited for this study, thus results are awaited.

Our preclinical results showed a synergistic effect of the CDK4/6 inhibitor ribociclib with PI3K inhibitor alpelisib in two HNSCC cell lines. These results need to be confirmed in larger (clinical) studies.

2.2 PI3K/ AKT/ MTOR inhibitors

Recent molecular characterization showed that in HNSCCs the PI3K/Akt/mTOR pathway, along with cell cycle, seems to be the most frequently deregulated cellular pathway, also involved in therapy resistance. PI3Ks are a class of enzymes vital for nutrient uptake, anabolic reactions, cellular growth, differentiation, and survival. They are activated by

receptor tyrosine kinases (RTKs), for example the epidermal growth factor receptor (EGFR).

In **Chapter 4** HPV positive and HPV negative HNSCC cell lines were treated with three different PI3K inhibitors (PI3Ki) (alpelisib, buparlisib and gedatolisib), which showed high efficacy to inhibit cell growth. PI3Ki treatment resulted in a downregulation of the proteins involved in the PI3K-Akt-mTOR pathway. Furthermore, an increase of cells in G1 and SubG1 after treatment with alpelisib and an increase of cells in G2/M phase after treatment with buparlisib was observed, whereas treatment with gedatolisib did only promote a subtle increase of cells in G1 phase in UPCI-SCC-03. All the three PI3K inhibitors induced a slight increase in apoptosis in all the cell lines, which was only statistically significant for buparlisib. We also found that PI3Ki treatment resulted in decrease of both the glycolysis and the mitochondrial oxidative metabolism in HNSCC cells.

The differences in results that we observed may be partly explained by the specific action of the inhibitors. Alpelisib, with the highest IC_{50}, selectively inhibits the alpha isoform of the PI3K catalytic subunit (p110α). In *HER2*-amplified and *PIK3CA* mutant luminal breast cancers, the initial efficacy of p110α inhibition is mitigated by rapid re-accumulation of the PI3K product PIP3 produced by the p110β isoform. The addition of a p110β inhibitor to alpelisib prevents the PIP3 rebound and induces greater antitumor efficacy in this luminal breast cancer study.[40] Treatment with buparlisib significantly inhibits wild-type and mutant PI3K catalytic subunit p110α, β, δ and γ and showed also in our study lower IC_{50} values than treatment with alpelisib. Gedatolisib is a highly potent dual inhibitor of PI3K (α, β, δ and γ) and mTOR (TORC1 and TORC2) and had the lowest IC_{50} values in our study. Despite the potent growth inhibitory effect of all three PI3Ki, with downregulation of the pathway and metabolic activity, studies are required to further explore their precise mechanism of action, also to explain why application of these inhibitors in the clinical setting has been so far disappointing.[41,42]

An explanation for this discrepancy could be the use of cell lines as preclinical models. In vitro human cell line models have been widely used to predict clinical response and to help identify novel mechanisms associated with variation in drug response. Furthermore, cell lines are well characterized and easy to handle in the laboratory, but there are also some disadvantages. The microenvironment and drug pharmacokinetic effects on clinical response can't be assessed.[43,44] Almost all HPV positive HNSCC cell lines are from smoking patients with aggressive tumor that failed to respond to initial therapy.[45,46] Therefore, the HPV positive HNSCC cell lines represent the HPV positive patients with a poor prognosis. Cell culturing can also introduce new mutations and can change the cell line characteristics letting them diverge from the primary tumor. A novel approach to

overcome limitations of in vitro cell line models could be the incorporation of three-dimensional (3D) primary cell culture models to better simulate the in vivo microenvironment. 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.[47-49]

These 3D models provide a more realistic way to grow tumor cells and allowing for interaction between different cell types. 3D models allow prediction of therapeutic response in a personalized setting and enable novel drug testing before introduction into clinical practice. So far, most information is available on HNSCC histocultures and their use to obtain an indication for response to chemotherapy. General disadvantages of histocultures and other 3D culture models are the difficulty to maintain them for longer period of time because of their limited lifespan and the possibility of central necrosis. Further improvement of these histocultures and/or other ex vivo tumor models is necessary in order to examine if they can mature further to useful clinical tool.[49]

A second explanation for the discrepancy between in vitro and in vivo results, is the activation of compensatory signaling pathways that bypass the efficacy of PI3Ki. Activation of PI3K leads to AKT-mediated phosphorylation of FOXO proteins. These proteins transcriptionally repress RTKs that activate PI3K, such as HER3, EGFR, insulin receptors and FGFRs.[50-52] Inhibition of PI3K blocks FOXO phosphorylation leading to an upregulation of RTKs that can again activate the PI3K-Akt-mTOR pathway. This activation of compensatory signaling pathways suggests that combinations of PI3K with agents against RTK signaling pathways, might be a more effective way to inhibit PI3K-Akt-mTOR pathway. However, so far, because of lack of selectively of these drug combinations and toxicity in patients, these treatments have been challenging.[52]

A third explanation for ineffective PI3Ki treatment in clinical trials may be the selection of patients recruited in the trials.[53] Selection of most patients is based on prior treatment failures and not on gene alterations underlying deregulation of the PI3K-Akt-mTOR pathway.[54,55] On the other hand, not all patients with for example *PI3KCA* mutations have similar benefit from PI3Ki. In a phase 1b trial, patients with *PI3KCA* mutations and concurrent alterations in *KRAS, TP53* or *FGFR1* did not benefit from alpelisib treatment.[52,56] Furthermore, in most studies, there are small number of patients without detectable *PIK3CA* mutations that respond clinically to PI3Ki.[52] Thus, more studies are needed to investigate if there is room for PI3Ki or combinations with other drugs in the treatment of HNSCC. Clinical trials with PI3K, AKT and mTOR inhibitors in HNSCC patients have only been completed up to phase II until now.[57]

2.3. Alternative treatment options in HPV associated OPSCCs

Despite the fact that HPV positive OPSCC have a prognostically favorable biological behavior, this has not resulted in different treatment strategies for HPV positive and negative OPSCCs. However, alternative therapies are needed for patients with HPV associated OPSCCs with reduced toxicity profiles and maintaining oncologic outcomes.

Several de-escalation therapy trials have been performed for HPV positive OPSCCs. Recently, two large phase III trials, RTOG 16[58] and De-ESCALaTE[59], attempted to reduce toxicity by replacing concurrent radiotherapy and cisplatin with radiotherapy and the use of cetuximab. Treatment with radiotherapy and cetuximab showed inferior overall survival and progression free survival and increased rates of locoregional failure. Therefore is it not advised to replace the standard regimen of concurrent chemoradiation therapy being cisplatin-radiotherapy.[60]

Another alternative treatment is the reduction in radiotherapy dose following induction chemotherapy. The phase II Optima-II trial, in which induction chemotherapy was followed by reduced-dose radiotherapy resulted in equal tumor control and less toxicities (grade 3 mucositis, dermatitis, and need for enteral feeding) in HPV positive patients treated with the reduction-dose of radiotherapy. These data are promising and should be translated to phase III studies.[61] Despite these de-escalation attempts, the current practice guidelines do not recommend de-escalation treatment for HPV positive HNSCC patients due to lack of evidence.[62]

Other alternative therapeutic approaches for HPV positive OPSCC patients were investigated in this thesis. One hypothesis was that the antiviral agent Cidofovir (CDV) could be effective in the treatment of HPV associated OPSCCs. 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.[63,64] Interestingly CDV has also shown an effect in the treatment of diseases caused by HPV, a virus which does not express its own DNA polymerase.[65,66]

In **Chapter 5** we indeed found that the cell growth of HPV positive and also HPV negative HNSCC cell lines was inhibited by CDV. Remarkably, in both cases, the host DNA polymerase is used for replication. This let us to investigate the mechanism underlying CDV inhibition in these cell lines. We showed that treatment with CDV caused DNA damage by means of DNA double strand breaks and as a result the DNA damage response pathway became activated. With an inappropriate apoptosis machinery, the cells appeared to undergo mitotic catastrophe. Earlier studies have also indicated that an impaired DNA damage repair is responsible for the elevated radiosensitivity of HPV-positive tumor cells.[67,68] 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.[69]

CDV is already used off-label for the treatment of infections caused by other DNA viruses, including papilloma- and polyomaviruses, for example in patients with recurrent respiratory papillomatosis (RRP). From this experience, there have been concerns that cidofovir may produce adverse effects which is relevant for the use of CDV in OPSCC patients, too. Some adverse effects are known to be dose dependent, including nephrotoxic effects and neutropenia, and have been observed only with high-dose systemic administration of cidofovir to treat cytomegalovirus.[70] Another described adverse effect is the increased risk of malignant transformation in patients with RRP treated with intralesional CDV.[71] In an international retrospective study evaluating 275 patients treated with intralesional CDV injection, no clinical evidence was found for more long-term nephrotoxicity, neutropenia or laryngeal malignancies after the administration of intralesional cidofovir.[72] In a recent study of Hoesli et al, evaluating the safety of intralesional CDV for treatment of RRP, the intralesion injection of CDV was not associated with increased risk of dysplasia or carcinoma or other complications in 154 patients.[73] Therefore the clinical (intralesional) use of CDV in OPSCC patients could be feasible.

Another possible treatment option for HPV positive OPSCC could be a therapeutical vaccine. Prophylactic HPV vaccines target the viral infection itself by inducing neutralizing antibodies and are effective in preventing HPV to induce malignancies but are not effective in treating them.[74] Targeting human papillomavirus E6 and E7 by DNA, peptides and other vaccines has already demonstrated clinical efficacy in HPV driven dysplasia.[75-78] Most HPV-targeting DNA vaccines have been studied in the setting of cervical intraepithelial neoplasia (CIN). In a phase IIb clinical trial, VGX-3100 DNA vaccine encoding E6 and E7 in combination with DNA vaccine encoding IL-2 has shown promising clinical results in women with HPV16 and -18 associated CIN.[75] A phase I/II trial to assess the vaccine safety and anti-tumor efficacy in combination with PD-L1-blocking mAb Durvalumab is now recruiting HPV positive HNSCC patients (NCT03162224). The combination of E6-E7 immunization and inhibition of the PD1/PDL1 checkpoint, is expected to double the natural immune system response against the cancer.[79] There is also a phase I/II clinical trial using an mRNA vaccine targeting HPV E6 and E7 recruiting HPV positive HNSCC patients (NCT03418480). The results of these trials are expected in the coming years.

3. Potential markers for HNSCC prognosis and therapy, based on HPV integration

Despite improvements in detection and treatment of HNSCCs, the mortality rates have hardly decreased over the last decades. Especially patients with a recurrent and/or metastatic HNSCC have a poor survival. HPV positive patients without a history of tobacco and alcohol consumption have a more favorable prognosis, due to better treatment response and lower risk of recurrence disease.[80-82] Whether this is due to the molecular pathogenesis or related to age and better overall health of these patients remains to be studied in more detail. Because of the more favorable outcome of HPV positive OPSCC patients, HPV positive and HPV negative oropharyngeal carcinomas are classified as separate entities, which is included in the 8th edition of the American Joint Committee on Cancer TNM staging system in 2017. To determine the HPV-status of a patient p16 immunostaining is used as surrogate marker. However, there is a subgroup of HPV positive patients having a less favorable prognosis and a greater risk of recurrence, development of a second primary tumor, and active vascular invasion.[1,83] Thus, the presence of HPV in tumor cells does not predict the outcome in the individual patient. How HPV integration affects its host cell and whether HPV integration contributes to the prognosis in HNSCC will be the subject of this last part of the thesis.

So far, the use of HPV as a biomarker for dose de-escalation or changing of treatment modalities has not been proven, as was already discussed in paragraph 2.3. Starting from a transient HPV infection, the viral genome maintains as extra-chromosomal episomes. Eventually a persistent infection may lead to the integration of the HPV genome into the host cell genome.[84] The results of studies looking at the association of HPV integration and prognosis are controversial. For example, the study of Koneva et al. showed that patients with (RNAseq determined) integration positive oropharyngeal and oral cavity tumors had worse survival than patients with integration negative tumors.[85] In contrary, Pinatti et al. recently found that HPV integration was correlated with favorable disease-specific survival when compared to patients without integration. A possible explanation for these different outcomes could be different techniques being used to detect HPV integration in tumor tissue. Pinatti et al. used Detection of Integrated Papillomavirus Sequences (DIPS) PCR which detects virus-human DNA sequences.[86] Other techniques are Amplification of Papillomavirus Oncogene Transcripts (APOT) PCR (which detects virus-human RNA transcripts) and quantitative (q)PCR-based techniques (which are used to determine E6/E7 copy numbers in relation to E2). Also in situ hybridization (ISH) or Fluorescence In Situ Hybridization (FISH), which can visualize HPV DNA or RNA as well as viral integration at the single cell level in cells and tissues, are used.[84] Altogether, there are large differences in integration incidence between different studies possibly

(partially) caused by different detection methods.[87-90] Another explanation for the different outcomes of the studies could be that there are often tumors included from different anatomical locations and often no distinction is made between solely integrated HPV and the mixed form, in which episomal DNA is also present. In addition, it often concerns small patient groups. These aspect make it difficult to compare the different studies.

In **Chapter 6** we showed that OPSCCs with viral integration (determined with APOT PCR and DIPS PCR) showed deregulated expression of genes involved in the metabolic pathways, and we found particularly frequent upregulation of AKR1C1 and/or AKR1C3 expression. Upregulated AKR1C1 and AKR1C3 expression was associated with poor prognosis in HPV positive, but also in HPV negative tumors. Expression of these genes were a negative predictor in correlation to the outcome of chemo- and radiotherapy both in overall survival and disease-free-survival. If these results can be confirmed in larger studies, than AKR1C1 and AKR1C3 may be considered to be included in prediction models in OPSCCs. Low risk groups could then profit from de-intensification of treatment protocols, whereas intermediate and high risk groups could be selected for other therapeutic options.

AKR1Cs metabolize lipids, steroid hormones, retinoic acids and are phase 1 detoxifying enzymes in the degradation of pharmacological agents and tobacco smoke components. Upregulation of AKR1Cs prevents the accumulation of cytotoxic ROS, an important mechanism leading to resistance against chemotherapeutic drugs such as cisplatin.[91] AKR1Cs are deregulated in a broad range of chemoresistant human cancers (e.g. ovarian, cervical, liver and (non-small cell) lung cancer) and are promising target proteins with prognostic and therapeutical potential. Drugs that can inhibit AKR1C isoforms are e.g. nonsteroidal anti-inflammatory drugs (NSAIDs), pan-AKR1C inhibitors or indomethacin. In the Nurses Health cohort study, there was a prolonged survival of ovarian cancer patients using cisplatin in combination with use of NSAIDs.[92] In HNSCC cell lines, treatment of cisplatin resulted in a 3-4 fold overexpression of AKR1Cs in comparison with untreated controls.[93] In the study of Peraldo-Neia et al., pretreatment with a selective AKR1C3 inhibitor potentiated the effect of cisplatin in OPSCC cell lines exhibiting higher basal AKR1C3 expression levels.[94] However, due to the high expression of AKR1C enzyme in normal tissues like liver and pancreas, it will be important to investigate potential adverse events of these drugs in these tissues.

There are promising tumor biomarkers in development. The further development of these tumor markers has to be fostered in order to improve the stratification of patients for different therapy arms. The increase in the use of sequencing techniques allows the determination of the genetic background of a tumor and can help to identify affected signaling pathways which can be used for molecular treatment approaches.[95]

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Chapter 8

Summary

Summary

While the incidence of tobacco related cancer has declined in the past two decades, because of a reduction in the prevalence of smoking in most high-income countries, there is an increase in HPV associated oropharyngeal squamous cell carcinoma (OPSCC). More specifically, the prevalence of HPV in OPSCC increased from 21.4% in 2004 to 50% in 2011 in Maastricht University Medical Center. An HPV vaccine has been available for some time now, which may reduce not only the incidence of uterine cervical cancer but also HPV associated head and neck squamous cell carcinoma (HNSCC). In the Netherlands, the HPV vaccine is since 2010 available for girls and since 2022 for boys from the age of ten. In order to maximize the potential benefits of HPV vaccination, it is necessary to get the vaccination coverage as high as possible. Therefore, it is important that patients and health care professionals are aware of the human papillomavirus, the association of the virus with cancer and the availability of an HPV vaccination. Until now it was unclear what the knowledge is about the role of HPV in OPSCC under the general population and general practitioners in the Netherlands.

Chapter 2 shows the results of a study examining the awareness of HPV associated OPSSC among a representative sample of the Dutch population. 30.6% of the participants had heard of HPV and only 29.9% of these participants knew about the association between HPV and OPSCC. 49.7% of the participants knew that there is an HPV vaccine available. The results of this survey indicate that the public awareness of HPV and the association with OPSCC is lacking. The awareness about HPV, the HPV vaccine and the link of HPV with OPSCC was greater among women and suggest that this knowledge is primarily due to awareness of the role of HPV in the development of uterine cervical cancer. Greater awareness of the role of HPV infection in OPSCC is necessary to improve vaccine uptake, in women but especially also in men.

The awareness of the association between HPV and OPSCC is much higher under the general practitioners in the Netherlands (72%), but more than a quarter of them is unaware of HPV as a causative factor for OPSCC. The results in **chapter 3** show that there was limited awareness among the general practitioners regarding gender, age and prognosis of patients with HPV associated OPSCC. Only 35.5% of the participating general practitioners was aware that HPV associated OPSCC patients are more often male, and just over half of the participants knew that these patients are generally younger of age. Interventions to increase awareness of HPV and its association with non-uterine cervical cancer should be considered, which might help to increase the HPV vaccination uptake and earlier diagnosis of this disease leading to improved survival.

HPV vaccination and increased awareness of HPV related OPSCC are possibilities to improve the prognosis of these cancers. Most HPV positive tumours have a more favourable prognosis in comparison with HPV negative tumours, however a subgroup of HPV positive tumours shows less favourable prognosis with a greater risk of recurrence or developing a second primary tumor. In order to improve the prognosis of HNSCC patients, there are new therapeutical strategies necessary. There are different pathways whose deregulation play a role in the development of HPV positive and -negative HNSCC and thus may be potential targets in the treatment of HNSCC. Therefore, we studied whether or not medication targeting some of the most important pathways are able to improve treatment.

Because HNSCC is associated with activation of the PI3K/Akt/mTOR pathway and also with deregulation of the core cell-cycle regulatory machinery, we investigated the in vitro antiproliferative effects of several PI3K/Akt/mTOR pathway inhibitors (alpelisib, buparlisib and gedatolisib) and CDK4/6 inhibitors (palbociclib and ribociclib) in HPV positive and -negative HNSCC cell lines (**chapter 4)**. In addition, we compared the inhibitors with the growth inhibitory effect of cisplatin, which is the current, most widely used chemotherapeutical treatment option in HNSCC. PI3K inhibitors and CDK4/6 inhibitors proved to efficiently inhibit their respective pathways and HNSCC cell growth in vitro, the latter only in HPV negative cell lines. Whereas PI3K inhibition especially induces apoptosis and attenuates cellular metabolism, CDK4/6 inhibition particularly leads to cell cycle arrest. Further research should elucidate whether (a combination of) these inhibitors may be effective therapeutic agents for HNSCC patients.

Another possible therapeutic option for HNSCC is the antiviral agent Cidofovir (CDV), which is an acyclic nucleoside phosphonate targeting DNA viruses that encode for their own DNA polymerase. Besides direct antiviral effects in DNA viruses CDV has also demonstrated antiproliferative properties against HPV positive and HPV negative malignancies in vitro and in vivo. The molecular mechanism underlying the efficacy of CDV is not completely understood, as HPV uses the host DNA polymerase for replication. Therefore, in **chapter 5,** the antiproliferative effects of CDV were investigated in HPV positive and HPV negative HNSCC cell lines and the normal oral keratinocyte cell (NOK) cell line. We investigated whether the antiproliferative effect was caused by a difference in response to DNA damage. CDV inhibited the cell growth of all the HPV positive and -negative HNSCC cell lines. Treatment with CDV caused DNA damage by means of DNA double strand breaks (DSBs) and as a result the DNA damage response pathway became activated. There was more DNA damage visible in the HPV positive cell lines showing the strongest inhibition as compared to the HPV negative cell lines showing significantly less

inhibition by CDV. CDV treatment resulted in G2/M phase arrest, but apoptosis did not appear to occur. Rather our data indicate the occurrence of mitotic catastrophe.

Following an HPV infection, the virus can remain in its episomal form, or the HPV genome become eventually integrated into the host cell genome. So far, there is little evidence that viral integration may have impact on prognosis and it is unclear if there is a biological consequence of viral integration. Therefore in **chapter 6**, by comparing HPV16 positive OPSCC harboring episomal or integrated virus using mRNA microarray expression profiling, we identified a unique signature of differentially expressed human mRNAs in relation to viral physical state. The tumors with viral integration showed deregulated expression of genes involved in metabolic pathways, frequently including upregulated Aldo-keto-reductase 1C1 and/or 1C3 (AKR1C1 and AKR1C3) expression. Survival analysis of 141 additionally immunostained OPSCC (HPV positive as well as HPV negative) showed unfavorable survival rates for tumors with upregulation of AKR1C1 or AKR1C3.

If these results could be confirmed in larger studies, than AKR1C1 and AKR1C3 may be considered to be included in prediction models in OPSCC, independent of HPV status. Low risk groups (for example HPV positive OPSCC tumor without AKR1C upregulation) could then potentially profit from de-intensification of treatment protocols, whereas intermediate and high risk groups could be selected for other therapeutic options, such as inhibitors of the PI3K and NRF2 pathways including AKR1C.

Samenvatting

Hoewel de incidentie van tabak gerelateerde kanker de afgelopen twee decennia is afgenomen, vanwege een afname van de prevalentie van roken in de meeste welvarende landen, is er een toename van HPV-geassocieerde orofaryngeale plaveiselcelcarcinoom (OPSCC). Meer specifiek is de prevalentie van HPV in OPSCC gestegen van 21.4% in 2004 naar 50% in 2011 in het Maastricht Universitair Medisch Centrum. Er is al enige tijd een HPV vaccin beschikbaar, dat niet alleen de incidentie van baarmoederhalskanker kan verminderen, maar ook van HPV-geassocieerd hoofd-hals plaveiselcelcarcinoom (HNSCC). In Nederland is het HPV vaccin sinds 2010 beschikbaar voor meisjes en sinds 2022 voor jongens, voor beiden vanaf tien jaar. Om de potentiële voordelen van HPV-vaccinatie te maximaliseren, is het noodzakelijk om de vaccinatiegraad zo hoog mogelijk te krijgen. Daarom is het belangrijk dat patiënten en zorgverleners op de hoogte zijn van het humaan papillomavirus, de associatie van het virus met kanker en de beschikbaarheid van een HPV-vaccinatie. Tot nu toe was het onduidelijk wat de kennis is over de rol van HPV bij OPSCC onder de algemene bevolking en huisartsen in Nederland.

Hoofdstuk 2 toont de resultaten van het onderzoek naar de bekendheid van HPV-geassocieerde OPSSC onder een representatieve steekproef van de Nederlandse bevolking. 30.6% van de deelnemers had gehoord van HPV en slechts 29.9% van deze deelnemers wist van het verband tussen HPV en OPSCC. 49.7% van de deelnemers wist dat er een HPV vaccin beschikbaar is. De resultaten van dit onderzoek geven aan dat het publieke bewustzijn van HPV en de associatie met OPSCC ontbreekt. Het bewustzijn over HPV, het HPV vaccin en de link van HPV met OPSCC was groter bij vrouwen en suggereert dat deze kennis voornamelijk te danken is aan de kennis over de rol van HPV bij de ontwikkeling van baarmoederhalskanker. Een groter bewustzijn van de rol van HPV-infectie bij OPSCC is nodig om de vaccinatiegraad te verhogen, bij vrouwen maar vooral ook bij mannen.

Het besef van het verband tussen HPV en OPSCC is veel hoger onder de huisartsen in Nederland (72%), maar meer dan een kwart van hen is niet op de hoogte van HPV als oorzakelijke factor voor OPSCC. De resultaten in **hoofdstuk 3** laten zien dat huisartsen zich maar beperkt bewust waren van geslacht, leeftijd en prognose van patiënten met HPV geassocieerde OPSCC. Slechts 35.5% van de deelnemende huisartsen wist dat HPV geassocieerde OPSCC patiënten vaker mannelijk zijn en iets meer dan de helft van de deelnemers wist dat deze patiënten over het algemeen jonger zijn. Interventies om het bewustzijn van HPV en de associatie ervan met niet-baarmoederhalskanker te vergroten,

moeten worden overwogen, wat zou kunnen helpen om de vaccinatiegraad van HPV en een eerdere diagnose van deze ziekte te vergroten, wat kan leiden tot een betere overleving.

HPV vaccinatie en een groter bewustzijn van HPV gerelateerde OPSCC zijn mogelijkheden om de prognose van deze kanker te verbeteren. De meeste HPV positieve tumoren hebben een gunstigere prognose in vergelijking met HPV negatieve tumoren, maar een subgroep van HPV positieve tumoren heeft een minder gunstige prognose met een groter risico op recidief van de ziekte of het ontwikkelen van een tweede primaire tumor. Om de prognose van HNSCC patiënten te verbeteren, zijn nieuwe therapeutische strategieën nodig. Er zijn verschillende cellulaire signaalroutes (pathways) waarbij de deregulatie een rol speelt bij de ontwikkeling van HPV positieve en -negatieve HNSCC en dus mogelijke doelwitten kunnen zijn bij de behandeling van HNSCC. Daarom hebben we onderzocht of medicatie gericht op enkele van de belangrijkste signaalroutes (pathways) de behandeling kan verbeteren.

Omdat HNSCC geassocieerd is met activering van de PI3K/Akt/mTOR signaalroute (pathway) en ook met deregulering van de cel cyclus, hebben we de in vitro antiproliferatieve effecten van verschillende PI3K/Akt/mTOR remmers (alpelisib, buparlisib en gedatolisib) en CDK4/6 remmers (palbociclib en ribociclib) onderzocht in HPV positieve en -negatieve HNSCC cellijnen (**hoofdstuk 4**). Daarnaast hebben we de remmers vergeleken met het groei remmende effect van cisplatine, de huidige, meest gebruikte chemotherapeutische behandelingsoptie bij HNSCC. PI3K remmers en CDK4/6 remmers bleken hun respectieve signaalroutes en HNSCC celgroei in vitro efficiënt te remmen, de laatste alleen in HPV negatieve cellijnen. Terwijl PI3K remming vooral apoptose induceert en het cellulaire metabolisme remt, leidt CDK4/6 remming vooral tot het stoppen van de celcyclus. Verder onderzoek moet uitwijzen of (een combinatie van) deze remmers effectieve therapeutische middelen kunnen zijn voor HNSCC patiënten.

Een andere mogelijke therapeutische optie voor HNSCC is het antivirale middel Cidofovir (CDV), een middel dat zich richt op DNA virussen die coderen voor hun eigen DNA polymerase. Naast directe antivirale effecten in DNA virussen heeft CDV ook antiproliferatieve eigenschappen aangetoond tegen HPV positieve en HPV negatieve maligniteiten in vitro en in vivo. Het moleculaire mechanisme dat ten grondslag ligt aan de werkzaamheid van CDV wordt niet volledig begrepen, aangezien HPV de DNA polymerase van de gastheer gebruikt voor replicatie. Daarom werden in **hoofdstuk 5** de antiproliferatieve effecten van CDV onderzocht in HPV positieve en HPV negatieve HNSCC cellijnen en de normale orale keratinocyten cel (NOK) cellijn. We onderzochten

of het antiproliferatieve effect werd veroorzaakt door een verschil in reactie op DNA schade. CDV remde de celgroei van alle HPV positieve en -negatieve HNSCC cellijnen. Behandeling met CDV veroorzaakte DNA schade door middel van DNA-dubbelstrengsbreuken (DSB's) en als gevolg daarvan werd de signaalroute voor DNA schade geactiveerd. Er was meer DNA schade zichtbaar in de HPV positieve cellijnen die de sterkste remming vertoonden in vergelijking met de HPV negatieve cellijnen die significant minder remming door CDV vertoonden. CDV behandeling resulteerde in ophoping van cellen in de G2/M-fase, maar apoptose leek niet op te treden. Onze resultaten wijzen eerder op het optreden van een mitotische catastrofe.

Na een HPV infectie kan het virus in zijn episomale vorm blijven, of het HPV genoom wordt uiteindelijk geïntegreerd in het genoom van de gastheercel. Tot nu toe is er weinig bewijs dat virale integratie invloed kan hebben op de prognose en het is onduidelijk of er een biologisch gevolg is van virale integratie. Daarom identificeerden we in **hoofdstuk 6**, door het vergelijken van HPV16 positieve OPSCC met episomaal of geïntegreerd virus met behulp van mRNA microarray-expressieprofilering, een unieke signatuur van differentieel tot expressie gebrachte menselijke mRNA's in relatie tot de virale fysieke toestand. De tumoren met virale integratie vertoonden gedereguleerde expressie van genen die betrokken zijn bij metabole routes, waaronder vaak opgereguleerde expressie van Aldo-keto-reductase 1C1 en/of 1C3 (AKR1C1 en AKR1C3). Overlevingsanalyse van 141 extra immuungekleurde OPSCC (HPV positief en HPV negatief) toonde ongunstige overlevingspercentages voor tumoren met opgereguleerde AKR1C1 of AKR1C3.

Als deze resultaten in grotere onderzoeken zouden kunnen worden bevestigd, dan zouden AKR1C1 en AKR1C3 kunnen worden opgenomen in OPSCC voorspellingsmodellen, onafhankelijk van de HPV status. Groepen met een laag risico (bijvoorbeeld HPV positieve OPSCC tumoren zonder AKR1C upregulatie) zouden dan potentieel kunnen profiteren van de-intensivering van behandelprotocollen, terwijl groepen met een gemiddeld en hoog risico zouden kunnen worden geselecteerd voor andere therapeutische opties, zoals remmers van de PI3K- en NRF2-signaalroutes, waaronder AKR1C.

Chapter 9

Impact

Impact

The results of the first study of this thesis show that there is a lack of knowledge among the Dutch population about the role of HPV in oropharyngeal cancer. In addition, only 49.7% of the study population knew of the existence of an HPV vaccine, despite the current vaccination programme against HPV related cervical and oropharyngeal cancer. If the knowledge about the role of HPV in the development of oropharyngeal cancer and the role of the HPV vaccine in the protection against these cancer types increases, hopefully the vaccination grade will increase with a decrease of HPV related cancers in the future.
The results of our study have therefore been used for the national 'Make Sense Campaign' (see attachment), which is a yearly initiative from the Dutch Working Group on Head and Neck Tumors (NWHTT), in order to create more awareness about head and neck cancer among the Dutch population and in this specific case about HPV related oropharyngeal cancer.

General practitioners (GPs) in The Netherlands are relatively well aware of HPV as a causative factor for oropharyngeal cancer, but there is a gap in knowledge on the characteristics of patients at risk for HPV associated oropharyngeal cancer. Further education on these subjects could improve disease recognition and thereby early treatment and patient survival. The GPs who participated in this study received a fact sheet with information about HPV and the role of HPV in oropharyngeal cancer. In addition, the study results have also been published as an infographic in the Dutch journal 'Huisarts & Wetenschap' (designed by Studio Wiegers, see attachment). As a consequence, we expect that the knowledge about HPV and oropharyngeal cancer among GPs in the Netherlands will increase, which will contribute to an earlier recognition of patients with head and neck cancers and to an earlier refer for further diagnosis and treatment.

In addition, there is a need to improve treatment for HNSCC, as the 5-year overall survival rate is still around 40-50%. In contrast to some other types of cancer, improving treatment for head and neck cancer remains a challenge because HNSCC is a heterogenous disease. Nevertheless, recently some promising targetable pathways for new therapeutic approaches have been identified. We tested several PI3K pathway- and CDK4/6 inhibitors for their efficacy to inhibit cell growth in HPV positive and negative head and neck cancer cell lines. The results of these studies were promising and may stimulate further research to bring these substances into clinical practice. Furthermore,

it should be tested whether or not it may be useful to combine these agents with each other as well as with radiotherapy.
We also tested the antiviral agent Cidofovir and showed that the working mechanism is different than supposed until now. Treatment resulted in DNA damage and mitotic catastrophe in the head and neck cancer cell lines, independent of HPV status. This mechanism is also seen in radiotherapy.
The results of this thesis were published in high impact scientific journals and when possible as open access articles in order to create transparency and to target a broad audience. The results have also been shared through presentations at (inter)national congresses, for example at the International Academy of Oral Oncology World Congress (2019, Rome) and the International Symposium on HPV-infection in Head and Neck Cancer (2022, Amsterdam).

Earlier research has addressed that, in general, HPV positive tumors have a far more favourable prognosis than HPV negative tumors. However, the presence of HPV in tumor cells does not predict outcome in the individual patient. A subgroup of HPV positive patients shows a less favorable prognosis with a greater risk of recurrence or development of a second primary tumor. Therefor de-intensification of treatment for HPV positive tumors has not been possible until now and the treatment of these two different subtypes of HPV positive cancers is still identical while the intensity of treatment is linked to the severity of side effects and loss of quality of life. A possible mechanism leading to differences in biological behaviour of different HPV positive tumors is viral genomic integration. However, very little is known about this until now. We have shown that oropharyngeal cancer with HPV integration show an altered expression of genes involved in cell metabolism, resulting in upregulation of the enzyme AKR1C. These tumors with increased expression of AKR1C show an unfavourable prognosis. With this insight we've delivered an additional potential factor for identifying HPV positive tumors in which treatment de-intensification can be applied. However, these results have to be confirmed in a larger group of patient. Furthermore, for tumors with an unfavourable prognosis a potential new therapeutic agent, AKR1C inhibitors, has been identified. This offers the possibility to start new (pre) clinical studies.

Make Sense Campaign

HPV and throat cancer

Recently, it has become clear that, in addition to smoking and alcohol consumption, human papillomavirus (HPV) is also a risk factor for the development of head and neck cancer, and in particular throat cancer.
An HPV infection is a sexually transmitted disease. Frequent sexual contact and oral sex are risk factors for HPV related throat cancer. Recent studies in the United States show that HPV related throat cancer appears to be more common than HPV related cervical cancer. Furthermore, patients with this form of throat cancer are more often male than female, are in relatively good health and do not smoke and/or drink excessively alcohol. Throat cancer occurs in the back of the throat, tongue or tonsils.

HPV vaccine for boys and girls

Since 2009, the HPV vaccine has been available in the Netherlands from the National Immunization Program for girls from 13 years of age. Since then, knowledge about HPV as a risk factor for cervical cancer among the population has increased. However, this did not translate into a high vaccination coverage: in 2019, only 53% of 13-year-old girls have been vaccinated. Since 2022, the HPV vaccine has also been available for boys. Girls and boys can be vaccinated with this vaccine from the age of nine.

Knowledge of HPV among the population

The percentage of the general population that is aware of HPV as a risk factor for throat cancer was unknown until now. For this reason we recently conducted a survey which revealed that 30.6% of those surveyed had heard of HPV. And 29.2% of those surveyed who had previously heard of HPV knew that HPV is a risk factor for throat cancer. That is only 11% of all respondents! We then also asked whether or not people were aware of the HPV vaccine: 49.7% indicated that they were aware of this vaccine.
We can therefore conclude that only a limited percentage of people knows that HPV is a risk factor for the development of throat cancer. Also, there was little knowledge about the availability of the HPV vaccine for boys and girls from 9 years old. It is therefore important that the Dutch population becomes more aware of the risks of HPV: for women the risk of developing cervical cancer and for men and women the risk of developing throat cancer. It is also essential that people are aware that the HPV vaccine can protect against these cancers. With increasing knowledge, we expect that more girls and boys will be vaccinated, which will finally lead to a decrease in incidence of these diseases.

Knowledge of HPV among General Practitioners (GPs)

In addition to a higher vaccination coverage, we also want a higher cure rate for HPV related throat cancer. Because the chance of recovery increases if patients are treated as soon as possible, it is important that GPs are aware of this form of throat cancer. In this way they can search for symptoms of the disease in a more targeted way and interpret them better/faster. That is why we also conducted a survey among the GPs in the Netherlands. The majority (72%) of GPs were found to be aware of HPV as a risk factor for throat cancer. 35% of the GPs knew that HPV related throat cancer is more common in men and 50% were aware that this form of throat cancer occurs at a relatively younger age.

These results of the survey may be a result of the fact that a GP sees relatively few patients with throat cancer. Treatment for patients with head and neck cancer in the Netherlands is centralized. This means that specialists in the centres see large numbers of patients every year.

With more than 12,000 GPs in the Netherlands and just over 3,000 new patients with head and neck cancer every year, a general practitioner only sees a patient once every four years. And the group of patients with HPV related throat cancer is even smaller. We therefore find it essential that specialists actively share their knowledge with GPs. We have attempted to raise awareness of HPV related throat cancer among primary care physicians by conducting our survey and sending them the latest information on the subject.

Overall, GPs are relatively well aware of HPV as a risk factor for throat cancer. Detailed knowledge, for example that the disease is more common in younger men who do not smoke and/or drink alcohol excessively, can still be improved. Hopefully, our research and the information sent will contribute to an increase in this specific knowledge.

Conclusions

Not enough people in the Netherlands know that HPV is a risk factor for developing throat cancer.

GPs are relatively well informed about HPV as a risk factor for throat cancer. Detailed knowledge, for example that the disease is more common in younger men who do not smoke and/or drink alcohol excessively, can still be improved.

Drs. F. Verhees, ENT specialist Maastricht UMC+

Also on behalf of the research group: drs. I. Demers, prof. E.J.M. Speel and prof. B. Kremer

Infographic Huisarts & Wetenschap

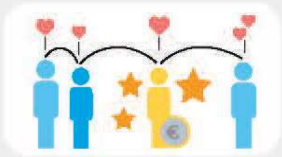
Oropharyngeal cancer caused by the human papilloma virus

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. This has roughly increased from 5 to 50% over the past 30 years. In Europe, on average 40-50% of all oropharyngeal carcinomas are associated with HPV infection.
However, the incidence of HPV negative head and neck tumors is decreasing.

Head and neck cancer is most often associated with the traditional risk factors of smoking and excessive alcohol consumption. These factors are the cause in 50% of the oropharynx carcinomas.

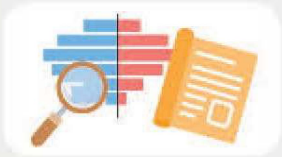


HPV positive tumors, on the other hand, are more common in (younger) patients without these traditional risk factors, but with varying sexual contacts and a higher socioeconomic status.

HPV positive oropharyngeal tumors present, more often than HPV negative tumors, as small (asymptomatic) tumors and are often already metastasized to the cervical lymph nodes.

Despite this, the prognosis for HPV positive tumors is generally better regardless of the therapy used (surgery, chemotherapy and/or radiotherapy).

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



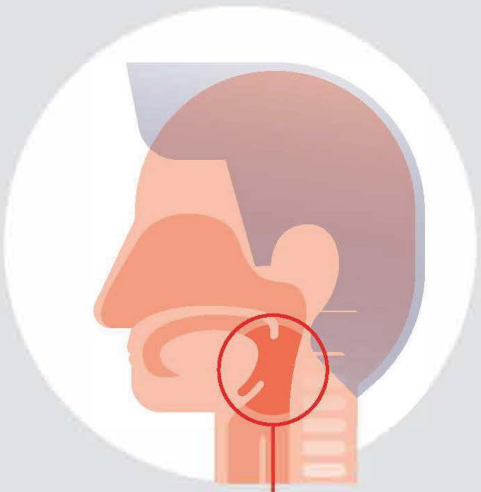
Sufficient knowledge about HPV positive oropharyngeal cancer and associated patient characteristics contributes to early recognition of the disease.

Due to the increasing incidence of HPV positive oropharyngeal cancer and the biological and clinical differences with HPV negative tumors, it is important that general practitioners are aware of this.

HUISARTS EN WETENSCHAP · JULI 2022

RESEARCH

About 3000 new head and neck cancer patients are diagnosed every year, which means that a general practitioner only sees a head and neck cancer patient once every 4 years. The group with HPV related oropharyngeal cancer is even smaller. It is therefore important that medical specialists actively share their knowledge with general practitioners. By publishing this study, we hope that the knowledge about HPV positive oropharyngeal cancer among general practitioners will increase.



Questionnaire survey among 900 general practices in the Netherlands.

Of all 207 participating GPs were,

informed of the link between HPV infection and the development of oropharyngeal tumors.

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 the HPV associated oropharyngeal cancer patients are more likely (75-80%) to be male

54% were aware that these patients often (58%) are < 60 years when getting the diagnosis.

It was striking that only 17% of the GPs were aware of the better prognosis of HPV positive tumors, compared to HPV negative tumors. The estimated 5-year survival is 80-90% in HPV positive tumors and 50% in HPV negative tumors.

General practitioners in training or recently graduated general practitioners were more aware of the favorable prognosis than more experienced GPs (33% vs 43%)

In conclusion, it appears that a quarter of Dutch GPs are not aware of HPV as a risk factor for oropharyngeal cancer. In addition, the knowledge about patient characteristics can still be improved. Further training for general practitioners will contribute to an increased level of knowledge and better recognition of HPV positive oropharyngeal tumors.

Original article: Demers I, Verhees F, Schouten LJ, Muris JWM, Kremer B, Speel E-J. Orofarynxkanker veroorzaakt door het humaan papillomavirus. Huisarts Wet 2022;65:DOI:10.1007/s12445-022-xxxx-x. This is an edited translation of: Demers I, Verhees F, Schouten LJ, Muris JWM, Kremer B, Speel EJM. Awareness of HPV-associated oropharyngeal cancers among GPs in The Netherlands: a cross-sectional study. BJGP Open 2022;6(1):BJGPO.2021.0080.

Dankwoord

Dankwoord

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Curriculum vitae

Curriculum vitae

Femke Verhees werd op 8 november 1989 geboren te Breda. Zij behaalde haar VWO diploma in 2008 aan het Theresialyceum te Tilburg. Vervolgens begon zij aan de studie Geneeskunde aan de Universiteit Maastricht, die zij afrondde begin 2015. Tijdens haar wetenschappelijke stage in het laatste jaar van de Geneeskunde studie werd de basis gelegd voor dit proefschrift. Vervolgens startte zij in maart 2015 met de opleiding tot KNO-arts aan het MUMC+ (opleiders Dr. Janny Hof en Prof. Dr. Bernd Kremer) waarbij twee perifere stages werden gevolgd in het Elkerliek Ziekenhuis te Helmond (opleider dr. Paul Schuil) en in het Catharina Ziekenhuis te Eindhoven (opleider dr. Frank Adriaansen). Aansluitend volgde zij een fellowship otologie in het MUMC+. Sinds februari 2021 is zij werkzaam als KNO-arts in het MUMC+ en de Annadal Kliniek.