Aus der Klinik und Poliklinik für Hals-, Nasen- und Ohrenheilkunde der Universität zu Köln
Direktor: Universitätsprofessor Dr. med. Jens Peter Klußmann

Analysis of Expression and Regulation of AKR1C2 in HPV-Positive and -Negative Oropharyngeal Squamous Cell Carcinoma

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Maria Ziogas
aus Mainz

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1. Gutachter: Universitätsprofessor Dr. med. J. P. Klußmann
2. Gutachterin: Universitätsprofessorin Dr. rer. nat. J. George

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Herr Privatdozent Dr. rer. nat. Christian U. Hübbers, AG Molekulare Kopf-Hals-Onkologie der Klinik für Hals-, Nasen-, Ohrenheilkunde der Uniklinik Köln.
Weitere Personen waren an der Erstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich nicht die Hilfe einer Promotionsberaterin/eines Promotionsberaters in Anspruch genommen. Dritte haben von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertationsschrift stehen.

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Die in dieser Arbeit angegebenen immunhistochemischen Experimente zur Detektion von AKR1C2 sind nach entsprechender Anleitung durch Herrn PD Dr. Christian Hübbers und Herrn Dr. Oliver Siefer aus der AG Molekulare Kopf-Hals-Onkologie der Klinik für Hals-, Nasen-, Ohrenheilkunde der Uniklinik Köln von mir selbst ausgeführt worden. Die immunhistochemischen Daten bezüglich AKR1C1, AKR1C3 und Nrf2 wurden bereits vor Beginn meiner Promotionsarbeit durch Herrn Dr. Oliver Siefer und Herrn PD Dr. Christian Hübbers erhoben. Der in dieser Arbeit verwendete Patienten-Datensatz wurde ohne meine Mitarbeit durch die Klinik und Poliklinik für Hals-, Nasen- und Ohrenheilkunde der Universität zu Köln bereitgestellt und durch Frau Dr. Nora Würdemann erhoben. Die Bereitstellung der entsprechenden Paraffin-Blöcke erfolgte durch Frau Prof. Dr. Uta Drebber aus dem Institut der Pathologie der Uniklinik Köln. Die lichtmikroskopischen Aufnahmen wurden durch mich erstellt und die Quantifizierung der immunhistochemischen Signale wurde durch mich selbst durchgeführt. Der Datensatz wurde durch mich selbst mit den Programmen SPSS für Mac (IBM Software, 174 Armonk, NY, USA) und GraphPad Prism 6.0 (GraphPad Software, La 182 Jolla, California, USA) ausgewertet.
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Table of Contents

Abbreviations

AJCC	American Joint Committee on Cancer
AKR	aldo-keto-reductase
ARE	antioxidant response element
CDDP	cis-diamminedichloroplatinum(II) "Cisplatin"
CDK4	cyclin-dependent kinase 4
COX-2	cyclooxygenase-2
CPS	Combined Positive Score
CRT	chemoradiotherapy
CT	computed tomography
CUL3	Cullin 3-dependent ubiquitin ligase
E1 to E7	"Early genes" 1 to 7
E2F	E2 factor-Induces S-phase-specific genes as a transcription factor
E6AP	E6-associated protein
EGFR	epidermal growth factor receptor
EpRE	electrophilic response element
ERRα	estrogen-related receptor α
FDA	U.S. Food and Drug Administration
5-FU	5-fluorouracil
GABA	gamma-aminobutyric acid
GFP	Green Fluorescent Protein
GLOBOCAN	Global Cancer Observatory
HEK293	Human Embryonic Kidney 293 (cell line)
HNSCC	Head and neck squamous cell carcinomas
HPV	human papillomavirus
HR-HPV	HIGH-RISK human papillomavirus
HSPGs	heparan sulfate proteoglycans
hTERT	human telomerase reverse transcriptase
IARC	International Agency for Research on Cancer
ICTV	International Committee on Taxonomy of Viruses
IHC	immunohistochemistry
INK4a/ARF	inhibitor of cyclin-dependent kinase 4a/ alternative reading frame
ISH	in-situ hybridization
Keap1	Kelch-like ECH-associated protein 1
L1 to L2	"Late genes" 1 to 2
LCR	long control region
MAP	mitogen activated protein
Mdm2	murine double minute 2 - an E3 ubiquitin ligase
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor ‘kappa light chain-enhancer of activated B-cells

NNAL	nitrosamine alcohol
NNK	nicotine-derived nitrosamine ketone
NQO1	NADPH oxidoreductase
Nrf2	nuclear factor (erythroid-derived 2)-like 2
NSAID	nonsteroidal anti-inflammatory drug
OPSCC	oropharyngeal squamous cell carcinoma
ORF	open reading frame
ORI	origin of replication
OS	overall survival
p16	p16INK4a - Inhibitor of cyclin-dependent kinases (CDKs) 4 & 6
p53	tumor suppressor p53
PD-L1	programmed death-ligand 1
PET-CT	positron emission tomography – computed tomography
PGC-1α/ERRα	proliferator-activated receptor gamma co-activator 1α/estrogen-related receptor α
PG	prostaglandin
PI3K/AKT	phosphatidylinositol-3-kinase/protein kinase B
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PML	promyelocytic leukemia nuclear bodies
pRb	retinoblastoma protein
ROS	reactive oxygen species
RT	radiotherapy
SOD1	Superoxide Dismutase 1
SP1	specificity protein 1 - transcription factor
TP53	gene of the tumor suppressor protein p53
UICC	Union for International Cancer Control
USDC	ursodeoxycholic acid

1. Summary

Oropharyngeal squamous cell carcinoma (OPSCC) is a significant subtype of head and neck squamous cell carcinoma (HNSCC) with increasing global incidence, largely due to human papillomavirus (HPV) infection. HPV-positive OPSCC differs epidemiologically and clinically from its HPV-negative counterpart, exhibiting better prognosis and treatment response. Notwithstanding the advances that have been made in the fields of diagnosis and treatment, OPSCC continues to be associated with a high mortality rate. A subset of HPV-positive tumors, despite generally having a more favorable outlook, can exhibit poor prognosis similar to their HPV-negative counterparts. However, this subgroup is still not adequately characterized by diagnostic markers.

Oxidative stress has emerged as a crucial factor in OPSCC pathogenesis, influencing tumor progression, therapy resistance, and prognosis. Reactive oxygen species (ROS) contribute to genomic instability, promoting malignant transformation. Aldo-keto reductases (AKRs), particularly AKR1C2, play a pivotal role in oxidative stress response and steroid metabolism in OPSCC.

The objective of this research is to examine potential differences in AKR1C2 expression between HPV-positive and HPV-negative OPSCC, as well as within clinically significant subgroups of each. Furthermore, the study seeks to determine how these expression variations relate to patient outcomes in a series of 51 OPSCC tumor samples with known HPV status. In addition, in vitro experiments were conducted using HEK293 cell line models overexpressing the HPV16 E6*I splice variant to investigate its impact on AKR1C transcription and the activation of oxidative stress pathways.

Upregulated AKR1C2 expression is associated with a poor prognosis both in HPV-positive and HPV-negative OPSCC. Notably, AKR1C2 expression exhibits sex-specific effects, correlating with improved survival in female patients but poorer outcomes in males when increased. However, the expression of AKR1C2 showed no correlation with Nrf2 expression or with the expression of its family members, AKR1C1 and AKR1C3. Unlike the established regulation of AKR1C1 and AKR1C3, in vitro studies show that increased E6*I expression does not lead to a corresponding increase in AKR1C2 levels.

These findings underscore the complex interplay among AKR1C2, HPV, and patient sex, highlighting the need for personalized treatment strategies for OPSCC. Targeted inhibition of AKR1C2, considering sex-specific differences, may enhance therapeutic outcomes. Future research should investigate these mechanisms to enhance treatment efficacy.

Zusammenfassung

Das oropharyngeale Plattenepithelkarzinom (OPSCC) ist eine bedeutende Unterform des Plattenepithelkarzinoms im Kopf- und Halsbereich (HNSCC) mit weltweit steigender Inzidenz,

die größtenteils auf eine Infektion mit dem humanen Papillomavirus (HPV) zurückzuführen ist. HPV-positive OPSCC unterscheiden sich epidemiologisch und klinisch von HPV-negativen OPSCC und weisen eine bessere Prognose und ein besseres Ansprechen auf die Behandlung auf. Trotz der Fortschritte, die in den Bereichen Diagnose und Behandlung erzielt wurden, ist das OPSCC weiterhin mit einer hohen Sterblichkeitsrate verbunden. Obwohl HPV-positive Tumoren im Allgemeinen mit einer besseren Prognose assoziiert sind, zeigt eine bestimmte Untergruppe dieser Tumoren ein ähnlich ungünstiges Krankheitsverhalten wie HPV-negative Tumoren. Die diagnostische Charakterisierung dieser Untergruppe ist jedoch bislang unzureichend, da spezifische Marker fehlen, die eine eindeutige Identifikation ermöglichen.
Oxidativer Stress hat sich als entscheidender Faktor in der Pathogenese des OPSCC erwiesen, der das Fortschreiten des Tumors, die Therapieresistenz und die Prognose beeinflusst. Reaktive Sauerstoffspezies (ROS) tragen zur genomischen Instabilität bei und fördern die maligne Transformation. Aldoketo-Reduktasen (AKRs), insbesondere AKR1C2, spielen eine zentrale Rolle bei der Reaktion auf oxidativen Stress und dem Steroidstoffwechsel bei OPSCC.
Ziel dieser Arbeit ist es, potenzielle Unterschiede in der AKR1C2-Expression zwischen HPV-positiven und HPV-negativen OPSCC sowie innerhalb klinisch signifikanter Untergruppen innerhalb beider Gruppen zu untersuchen. In diesem Rahmen soll ermittelt werden, wie diese Expressionsunterschiede mit den Ergebnissen der Patienten in einer Reihe von 51 OPSCC-Tumorproben mit bekanntem HPV-Status zusammenhängen. Darüber hinaus wurden In-vitro-Experimente mit HEK293-Zelllinienmodellen durchgeführt, die die HPV16 E6*I-Spleißvariante überexprimieren, um ihre Auswirkungen auf die AKR1C-Transkription und die Aktivierung von oxidativen Stresswegen zu untersuchen.
Eine erhöhte AKR1C2-Expression ist sowohl bei HPV-positivem als auch bei HPV-negativen OPSCC mit einer schlechten Prognose verbunden. Bemerkenswert ist, dass die AKR1C2-Expression geschlechtsspezifische Auswirkungen hat und mit einer verbesserten Überlebensrate bei weiblichen Patienten, aber schlechteren Ergebnissen bei männlichen Patienten korreliert, wenn sie erhöht ist. Die Expression von AKR1C2 zeigte jedoch keine Korrelation mit der Nrf2-Expression oder mit der Expression seiner Familienmitglieder AKR1C1 und AKR1C3. Im Gegensatz zur etablierten Regulierung von AKR1C1 und AKR1C3 zeigen In-vitro-Studien, dass eine erhöhte E6*I-Expression nicht zu einem entsprechenden Anstieg des AKR1C2-Spiegels führt.
Diese Ergebnisse unterstreichen das komplexe Zusammenspiel zwischen AKR1C2, HPV und dem Patientengeschlecht, und verdeutlichen somit den Bedarf an personalisierten Behandlungsstrategien für OPSCC. Eine gezielte Hemmung von AKR1C2 unter Berücksichtigung geschlechtsspezifischer Unterschiede könnte die therapeutischen

Ergebnisse verbessern. Zukünftige Studien sollten diese Mechanismen weiter erforschen, um die Behandlung effektiver zu gestalten.

2. Introduction

2.1. Oropharyngeal squamous cell carcinoma (OPSCC)

2.1.1. Squamous cell carcinoma of the head and neck region

Malignant lesions of the upper aerodigestive tract are a heterogeneous group of different origins. Most of these are squamous cell carcinomas arising from the squamous epithelial lining of the oral cavity, oropharynx, hypopharynx, and larynx. Head and neck squamous cell carcinoma (HNSCC), in general, is the sixth most common cancer worldwide, with an estimated 878,000 new cases reported in 2022. HNSCC accounts for more than 90% of all head and neck cancers and presents a mortality rate of approximately 450,000 deaths per year by 2022 [1]. Oropharyngeal squamous cell carcinoma (OPSCC) is a subtype of HNSCC that develops in the epithelium of the tonsils, base of the tongue, soft palate, and pharyngeal walls.

2.1.2. Epidemiology of OPSCC

OPSCC is the only subgroup of HNSCC with an increasing incidence in recent years. The Global Cancer Observatory (GLOBOCAN) has projected a further rise in incidence, with an estimated 30% increase by 2030. This suggests an incidence of 1,08 million new cases annually by then [2,3].

This projected increase is driven mainly by changes in population demographics, including aging, as well as ongoing shifts in risk factor patterns, particularly the increasing prevalence of human papilloma virus (HPV) [4,5]. Thus, OPSCC can be classified into two categories based on etiology: HPV-positive and HPV-negative. This distinction is critical owing to their different epidemiological, clinical, and prognostic characteristics.

The incidence of HPV-positive OPSCC has been rising significantly, particularly in high-income countries, such as the United States, Canada, and parts of Europe. In these regions, HPV-positive OPSCC now account for the majority of oropharyngeal cancers [5]. Moreover, this increase is largely attributed to HPV16, which is responsible for approximately 90% of HPV-associated OPSCC cases [6]. The shift towards HPV-related OPSCC is linked to changes in sexual behaviors, such as increased oral sex practices and having multiple sexual partners, which facilitate the transmission of HPV [5]. However, the rise in incidence in the US is more pronounced in male patients [7], whereas in Germany, a higher increase has been reported in

female patients [8]. Further data analysis has shown a modest positive association of ever- and long-duration marijuana use with oropharyngeal cancer risk among HPV16 seropositive individuals [9]. In comparison, the prevalence of HPV-positive OPSCC in Asia and Latin America is lower than that in Western countries.

HPV-positive OPSCC presents distinct epidemiological characteristics, including a relatively younger age at diagnosis, higher socioeconomic status, and a lack of traditional risk factors such as smoking and heavy alcohol consumption. These patients generally have a better prognosis and response to treatment than their HPV-negative counterparts, with a 5-year survival rate exceeding 80% in many studies [10,11].

In contrast, OPSCC has been traditionally associated with heavy tobacco and alcohol use, which remain the predominant risk factors for HPV-negative OPSCC. Despite declining smoking rates in many parts of the world, HPV-negative OPSCC continues to contribute significantly to the global cancer burden, particularly in regions with high tobacco and alcohol consumption rates. Furthermore, HPV-negative OPSCC tends to occur in older patients. These cancers are often more aggressive and have a poorer prognosis, with a 5-year survival rate of generally less than 50% [12].

OPSCC diagnoses exhibit a substantial gender disparity, with males constituting approximately 70% of the cases and females constituting 30%. The incidence rate among men is notably higher, ranging from three to five times that of women [13], particularly in the 40-60 year age group. This translates to approximately 86,000 new cases among males and 20,000 new cases among females reported by 2022. During the same period, the mortality rate reached almost 43,000 male and 9,500 female patients [1].

Despite recent advancements in treatment, the global mortality rate of OPSCC remains high. Only approximately 50% of patients survive the first five years following diagnosis [14,15]. However, studies have indicated that HPV-associated OPSCC generally has a much better prognosis than HPV-negative OPSCC [16]. For instance, the RTOG0129 study by Ang et al. reported a 3-year overall survival (OS) rate of 82.4% for HPV-associated OPSCC, whereas the OS rate for classical OPSCC was only 57.1% [12]. The study also suggests the need for additional risk classification based on smoking status. Owing to the distinct clinical features and implications for treatment decisions, the current 8th edition of the TNM staging system of the American Joint Committee on Cancer/ Union for International Cancer Control (AJCC/UICC) separates p16-positive (and thus usually HPV-positive) OPSCC from p16-negative OPSCC. For HPV-associated/p16-positive OPSCC, the pN status is determined solely by the number of affected lymph nodes, regardless of the size of the lymph node metastasis or the presence of extranodal tumor spread [17].

2.1.3. Etiology and risk factors of OPSCC

In recent decades, numerous well-conducted studies have examined the increasing importance of HPV infection as a risk factor for OPSCC, coinciding with the rising global incidence of this cancer type [2,3,7,18,19].

The involvement of HPV in carcinogenesis within the head and neck region was first reported in 1983, based on histopathological characteristics and immunohistochemical staining of viral structural proteins [20]. In 1985, viral DNA from HPV types 11 and 16 was detected in head and neck squamous cell carcinomas (HNSCCs) by Southern blot analysis [21]. During the 1990s, studies confirmed the specific association between HPV and malignant neoplasms of the Waldeyer tonsillar ring [22,23]. Since then, numerous molecular and epidemiological studies have established a causal link between HPV infection and oropharyngeal squamous cell carcinoma (OPSCC) [24-27]. In 2007, the International Agency for Research on Cancer (IARC) officially recognized high-risk HPV (HR-HPV) infection as an independent risk factor for carcinogenesis in this anatomical region [28]. The viral oncoproteins, E6 and E7, drive the cell cycle and induce apoptosis. Transformation primarily occurs in basal cells of the lymphoepithelial crypt epithelium. In HPV-infected cells, E7 disrupts the binding between retinoblastoma protein (pRb) and transcription factor E2F, promoting the transcription of S-phase-specific genes and leading to the overexpression of the p16 protein. The E6 oncoprotein mediates ubiquitination and subsequent proteolytic degradation of the tumor suppressor protein p53 [29,30].

In contrast, HPV-negative HNSCCs arise from chronic exposure to exogenous noxious agents, and are primarily characterized by the accumulation of genetic and epigenetic alterations. Carcinogenesis is a multistage process driven by the activation of oncogenes and inactivation of tumor suppressor genes. Early mutagenic events often include deletions of chromosomes 3p and 9p. The loss of the INK4a-ARF locus on 9p21 results in the deletion of the tumor suppressor gene p16 [31]. In summary, the inactivation of the p53 and p16-cyclinD1-Rb signaling pathways serves as a functional equivalent in both HPV-positive and HPV-negative HNSCCs, representing a key early event in their respective carcinogenesis [3,5]. In HPV-negative HNSCCs, mutations frequently occur in the TP53 (72%) and INK4a-ARF (32%) gene loci, which code for p53 and p16INK4a proteins, respectively, whereas HPV-associated carcinomas typically retain the wild-type versions of these genes [31].

2.1.4. Diagnostics, therapy, and prognosis

The symptoms of oropharyngeal tumors often manifest late and are related to the anatomical location of the tumor. Common initial complaints include difficulty swallowing, ear pain (otalgia), and altered speech, which may be described as slurred or thick. The initial indication for cancer spread is frequently the enlargement of lymph nodes in the cervical level II region, located near the angle of the mandible. This swelling is often detected by visual inspection or

physical examination [32,33]. In HPV-related OPSCC, metastases to the cervical lymph nodes frequently occur as cystic structures [34]. Tumors that spread to the palatal arches, soft palate, or base of the tongue significantly worsen prognosis and can lead to trismus (lockjaw) and foetor ex ore, particularly when the pterygoid muscles are involved. In cases where the tonsil is affected, a painless ulcer may initially form, which can progress to ulcerated disintegration of the tonsil [35,36]. Oropharyngeal tumors predominantly occur in the tonsils and tongue base, accounting for 96% of the cases [19]. Furthermore, high-risk HPV infection is more frequently linked to tonsillar squamous cell carcinoma (TSCC).

OPSCC patients typically present with small primary tumors (classified as T1 or T2) along with nodal metastases. Furthermore, the symptoms of OPSCC can be easily confused with those of non-malignant conditions, such as globus pharyngeus or laryngopharyngeal reflux, leading to potential misdiagnosis. Consequently, when individuals exhibit neck masses without symptoms, it is crucial to assess them using diagnostic techniques, such as confirmatory ultrasound and fine-needle aspiration biopsy to facilitate precise diagnosis and prompt treatment [32].

Biopsies taken during endoscopy of the upper aerodigestive tract are performed to confirm OPSCC histologically. Imaging techniques such as sonography and computed tomography (CT) assess the nodal (N) and metastatic (M) status [36], whereas PET-CT and Magnetic Resonance Imaging (MRI) further evaluate tumor extent and metastasis [5]. To determine HPV status, PCR-based detection of viral DNA or in situ hybridization (ISH) targeting E6 and E7 mRNAs is combined with immunohistochemical (IHC) staining of the p16 protein. This method utilizes p16INK4a as an indicator of transcriptionally active high-risk HPV infection in both primary tumors and metastatic lymph nodes [37]. In German-speaking countries, surgical approaches are favored over radiation therapy for early stage OPSCC classified as cT1-2 N0, regardless of the human papillomavirus (HPV) status. Specifically, transoral laser surgery and transoral robotic surgery are the preferred treatment methods. Advanced OPSCC is typically treated with open surgery and reconstruction followed by postoperative radiotherapy (RT) or chemoradiotherapy (CRT) if necessary. Alternatively, definitive CRT, which includes 66–70 Gy of RT with concurrent platinum-based CRT, is a common approach [38]. Internationally, treatment practices vary, with RT or CRT often considered equivalent to primary surgery.

Given the typically more favorable prognosis for HPV-related OPSCC, there is an ongoing discussion about potentially reducing the intensity of treatment. As a result, several efforts have been made to reduce the intensity of CRT, which is the standard non-surgical treatment. However, any reduction in treatment intensity must be carefully planned to avoid compromising the oncological outcomes. Planning Phase III trials for HPV-associated OPSCC presents significant challenges, as large sample sizes are needed to prove the non-inferiority of reduced-intensity protocols, especially given the generally favorable prognosis of the disease.

Various approaches have been investigated to reduce the treatment intensity in patients with HPV-associated OPSCC. These methods encompass the administration of radiation therapy in conjunction with cetuximab [39,40], decreasing the dosage of RT following surgery [4142], lowering the chemotherapy dose in CRT [43], and combining reduced-intensity RT with immune checkpoint inhibitors.

Nevertheless, 20–25% of HPV-positive OPSCC patients face poor outcomes due to factors like smoking, advanced nodal stage, epidermal growth factor receptor (EGFR) overexpression, and chromosomal instability [10,44-46]. For metastatic or recurrent cases in which curative treatment is not viable, systemic palliative treatment is based on performance status, comorbidities, and programmed death-ligand 1 (PD-L1) expression [38]. For PD-L1-positive tumors (Combined Positive Score (CPS) ≥ 1), CDDP with 5-fluorouracil (5-FU) and pembrolizumab is recommended, or pembrolizumab alone if CPS ≥ 20 and disease burden is low [47-49]. In PD-L1-negative cases (CPS < 1), cetuximab combined with cisplatin, 5-FU [50] , or docetaxel is suggested as the first-line therapy [51].

2.1.5. Primary and secondary prevention of HPV-related OPSSC

In 2006, the U.S. Food and Drug Administration (FDA) approved Gardasil®, the first HPV vaccine, which protects against HPV-16 and HPV-18 (associated with cancer) as well as HPV-6 and HPV-11 (causing genital warts). Cervarix® (targeting HPV-16 and -18) followed in 2007, and Gardasil9® (covering nine HPV types) became available in 2014. Initially recommended for girls aged 9–14 years to prevent cervical cancer, HPV vaccination was expanded to include boys [52]. High vaccination rates in Western countries have reduced cervical cancer incidence [53-55]; however, since OPSCC affects older demographics, it will take decades to confirm the effectiveness of HPV vaccination in preventing HPV-associated OPSCC. Further, vaccination has also been shown to reduce oral HPV infection, which is a possible surrogate marker for OPSCC prevention [56]. Modeling studies predict that a 50% vaccination rate could lead to a 50% decrease in OPSCC occurrence within 40 years, whereas 80% coverage might reduce the incidence to only 20% of its initial rate [57].

Unlike cervical cancer, there is no clear primary site for HPV infection or detectable precursor lesions for HPV-related OPSCC, limiting screening options. Current methods, such as tonsillar brush cytology and mouth rinses, show limited sensitivity, and oral HPV DNA measurement has not been proven predictive of OPSCC [58-58].

Antibodies against HPV16 oncoprotein E6 are highly sensitive and specific markers for the detection of HPV-positive oropharyngeal cancer. These antibodies can detect HPV-positive (cancer) cells years before diagnosis, making them promising for early detection [59-62]. Liquid biopsies, detecting HPV DNA in blood or plasma, are also highly sensitive and effective for

monitoring after therapy [60-62], although early HPV antibodies are less useful for predicting treatment success [63].

2.2. Human papillomavirus (HPV)

2.2.1. Papillomaviridae and their taxonomy

The human papillomavirus types have been classified and taxonomically assigned to the five genera alpha, beta, gamma, mu, and nu by means of phylogenetic analyses [29,64].

The major capsid protein L1 (Late Region) gene encodes the primary structural protein in the papillomavirus genome and is the most conserved open reading frame (ORF). According to the guidelines set by the International Committee on Taxonomy of Viruses (ICTV), papillomavirus can be classified into different genera, species, and types. A characteristic genetic feature of the viral genome is that the ORF5 reading frame is conserved between genes coding for early and late proteins [29].

HPVs of the genus Alpha-papillomavirus preferentially infect the mucosal regions of the anogenital area and mouth. They can cause benign (HPV-6, 7, 54, 61, 71), malignant (HPV-16, 18, 32), or benign and malignant (HPV-26, 34, 53) tumors. In Alpha-papillomaviruses, L1 and L2 are highly adapted to target mucosal epithelial cells, whereas, in other genera, these capsid proteins have sequence differences that affect their ability to infect different types of epithelial cells.

Beta (HPV-5, 9, 49) and gamma papillomaviruses (HPV-4) are characterized by missing ORF5, whereas nu papillomaviruses (HPV-41) contain multiple ORFs in their L1-gene area [29]. The Beta, Gamma, Nu, and Mu genera are primarily associated with cutaneous HPV types. High-risk HPV (HR-HPV) types, such as HPV16 and 18, are commonly found in highly altered lesions and can contribute to the malignant progression of epithelial tumors. Low-risk HPV (LR-HPV) types, such as HPV6 and 11, are primarily found in benign conditions, such as genital warts and laryngeal papillomatosis, and are rarely present in malignant tumors [65].

2.2.2. Structure of HPV

The icosahedral capsid, 55 nm in diameter, comprises 72 capsomeres and contains a double-stranded circular DNA genome organized in a chromatin-like complex associated with cellular histones. Of the 72 capsomeres, 12 are surrounded by five others, while 60 interact with six capsomeres, all exhibiting five-fold rotational symmetry. This suggests that each capsomere is likely a pentamer composed of five molecules of the major structural protein, L1 (55 kDa). The minor structural protein L2 is thought to be located at the center of the pentavalent capsomeres. The capsid lacks an outer membrane (Fig. 1)[29,65,66].

The papillomavirus genome is a closed, circular, double-stranded DNA molecule that ranges in size from 5,748 to 8,607 bp, depending on the virus species. Human papillomaviruses typically have a genome of approximately 8,000 base pairs. This DNA is complexed with cellular histone proteins to form a nucleosome-like supercoiled minichromosome structure. All papillomaviruses share a similar genomic organization, which is divided into two main regions: an early region that encodes proteins E1 to E8, produced early in the virus's life cycle and necessary for DNA replication, transcription, and cell transformation, and a late region containing the genes for the structural proteins L1 and L2, which are synthesized later. The early region includes multiple overlapping open reading frames, efficiently increasing the coding capacity through the use of alternative splice sites and start codons in different reading frames. The late region encodes capsid proteins L1 and L2.

Only one strand of DNA is transcribed, so all viral gene products are encoded on that single strand, unlike in evolutionary closely related polyomaviruses. Between the end of the late region and the start of the early region lies a roughly 1,000 base pair non-coding sequence known as the long control region (LCR). The LCR contains key viral regulatory elements, including promoters, enhancers, and the origin of replication (ORI). Viral genomes are transcribed from multiple promoters into various mRNA molecules that are differently spliced and partially overlap. The activity of these promoters is regulated by both viral and cellular factors, and is restricted to the differentiation of epithelial cells [29,65].

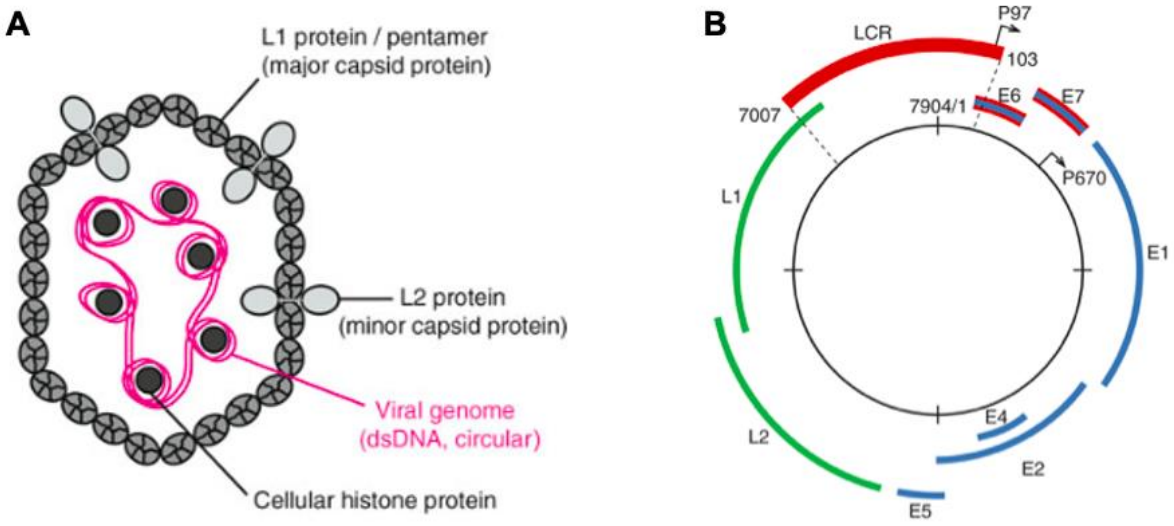

Fig. 1: **The structure and genome of the human papillomavirus (HPV)**

A The basic structure of an HPV particle. The outer shell (capsid) is composed of L1 proteins arranged in pentameric units (major capsid proteins) and interspersed with fewer L2 proteins (minor capsid proteins). Inside the capsid, the circular double-stranded DNA (dsDNA) genome (pink) is tightly associated with cellular histone proteins [67]**. B** Circular HPV16 genome from dsDNA (7,904 bp) transcribed clockwise**.** The **long control region (LCR)** contains regulatory elements, including the main viral promoter (P97), that drive early gene expression. Adjacent to this are the **E6** and **E7** oncogenes, which are critical for viral-mediated cell transformation. The **early genes** E2, E4, and E5 are involved in viral replication and regulatory functions. The **late genes** L1 and

L2 encode the major and minor capsid proteins, respectively. Numbering around the circle indicates the genomic coordinates, reflecting the circular nature of the dsDNA genome [68].

2.2.3. Viral infection cycle in the oropharynx

Human papillomavirus (HPV) gains access to epithelial cells through microabrasions or, in the case of high-risk HPV (HR-HPV), by infecting the single-layered squamous junction between the endocervix and ectocervix, or the basal membrane of the tonsillar crypt epithelium. It extends deeply into the lymphoid tissue of the tonsils, forming intricate branches that increase the surface area. It features a porous basement membrane and incomplete basal cell layer. This structural adaptation, particularly the reticulation of the squamous epithelium at the base of crypts, facilitates direct interaction between antigens and immunocompetent cells. Therefore, this region is particularly susceptible to HPV infection [69]. Successful infection depends on the binding of HPV to receptors on the basement membrane or surface of basal epithelial cells.

HPV initially attaches to host cells via its major capsid protein L1, which binds to heparan sulfate proteoglycans (HSPGs). This binding induces a conformational change in the viral capsid, exposing the L2 protein, which is then cleaved by proteases such as furin, allowing the virus to bind to secondary receptors, including EGFRs, integrins, and tetraspanins. After binding, HPV is internalized by the cell through macropinocytosis. It then moves through the cytoplasm, reaching the trans-Golgi network before entering the nucleus where it undergoes disassembly. The viral genome associates with promyelocytic leukemia (PML) nuclear bodies, which are essential for viral transcription and persistence [70].

In the nucleus, HPV initiates early transcription in basal cells, producing the E1 and E2 proteins required for initial genome replication. The E2 protein controls the expression of the E6 and E7 proteins, which are essential for the survival of infected cells and their entry into the S phase, which is a critical step for replicating the viral genome. As basal cells divide, they generate transit-amplifying cells, carrying viral genomes into the upper epithelial layers in alignment with epithelial differentiation [71].

HPV replication relies on S-phase host factors; E6 and E7 proteins support this by inhibiting cell cycle regulators, such as p53 and Rb, and promoting cell division during the lytic cycle [29]. During cell division, one daughter cell remains in the basal layer as a viral reservoir, whereas the other differentiates, losing its ability to divide. This cycle allows HPV to remain latent for several years.

2.2.4. HPV-induced carcinogenesis

Under typical physiological conditions, epithelial cells complete the final stage of their life cycle by exiting cell division and entering the quiescent phase (G0), following the post-mitotic G1

phase. However, the regulatory balance is disrupted in HPV-infected cells because of the actions of viral proteins E6 and E7. The E7 protein interferes with the retinoblastoma (Rb) pathway by binding to the Rb protein and its homologs p107 and p130. This interaction impairs the ability of the cells to stop the cell cycle at the G1/S checkpoint. In its hypophosphorylated form, Rb typically interacts with E2F transcription factors, inhibiting the expression of genes that are essential for entering the S phase. When Rb is phosphorylated by the cyclin-dependent kinase 4 (CDK4)/Cyclin-D1 complex, E2F is released, allowing for DNA synthesis and cell cycle progression. Simultaneously, E6 targets the tumor suppressor protein p53, which is a crucial regulator of the cell cycle and genomic stability. Under normal conditions, p53 levels are maintained low by its interaction with Mdm2, a ubiquitin ligase that marks p53 for degradation. Upon DNA damage, p53 is phosphorylated, diminishing its binding to Mdm2, leading to p53 accumulation and activation of genes responsible for cell cycle arrest or apoptosis. In HPV-infected cells, E6 binds to E6-associated protein (E6AP), enhancing the degradation of p53 and circumventing the cell's protective responses to DNA damage [29,30].

The combined effects of E6 and E7 undermine genomic stability and disrupt cell cycle control, resulting in an unchecked cellular proliferation. Interference in the pRb/E2F pathway by E7, coupled with the degradation of p53 facilitated by E6, leads to the accumulation of chromosomal abnormalities and an increase in centrosome number, which fosters an environment that supports malignant transformation [72,73].

Additionally, E6 also activates telomerases, such as human telomerase reverse transcriptase (hTERT), which is vital for maintaining telomere length. In normal cells, telomeres shorten with each cell division, leading to cellular aging and senescence. However, in HPV-infected cells, elevated telomerase activity supports unlimited cell replication, further promoting oncogenic transformation [29,74].

As a result, the expression of the cyclin-dependent kinase inhibitor p16 is markedly upregulated. This occurs because E7 inactivates pRb, thereby relieving the repression of p16 expression. As mentioned previously, overexpression of p16 serves as an indicator of HPV-associated cancers and is commonly used as a biomarker in clinical diagnostics [29,44].

2.3. Aldo-keto reductases

2.3.1. Oxidative stress and Nrf2-Keap1-CUL3 pathway

During aerobic respiration, signal transduction, inflammatory responses, and exposure to external factors, such as tobacco smoke, or environmental factors, such as UV radiation and reactive oxygen species (ROS), are continuously produced. ROS include free radicals, such as hydroxyl, superoxide, and peroxyl radicals (HO•, O2•, ROO•), as well as non-radical intermediates, such as hydrogen peroxide and singlet oxygen (H_2O_2, O_2). The human body detoxifies ROS through an antioxidant network consisting of non-enzymatic antioxidants (e.g., vitamins C and E, carotenoids, and thiols) and phase I and phase II enzymes involved in antioxidant and detoxification processes. Under normal conditions, balance or redox homeostasis is maintained between oxidative forces and antioxidative capacity of the body. When this balance shifts in favor of excessive ROS production, it leads to oxidative stress, which can result from both increased ROS formation and insufficient antioxidant defenses [75]. In this state, lipids, sugars, proteins, and nucleic acids undergo harmful oxidation leading to cellular damage. Accumulation of such damage is linked to various diseases, including cancer, cardiovascular disorders, and neurodegenerative conditions, and contributes to the aging process [76]. The NF-E2 p45-related factor, also known as nuclear factor (erythroid-derived 2)-like 2 (Nrf2), and Kelch-like ECH-associated protein 1 (Keap1) play an important role in inducing the intracellular response to oxidative stress. Nrf2, a Cap'n'Collar basic leucine zipper transcription factor, is regulated by binding to the cytosolic protein Keap1, which targets NRF2 for ubiquitination and proteasomal degradation. Keap1 acts as a substrate adaptor for E (CUL3). Keap1 is rich in cysteine residues, allowing it to sense ROS, electrophiles, Michael acceptors, and heavy metals. When these cysteine residues are oxidized or form adducts with electrophiles or metals, Keap1 undergoes a conformational change, which tightens its grip on Nrf2. This allows newly synthesized Nrf2 to escape Keap1-mediated ubiquitination and translocate to the nucleus. Once Nrf2 reaches the nucleus, it forms heterodimers with small Maf proteins and binds to the antioxidant response element (ARE), also known as the electrophilic response element (EpRE), in responsive genes (Fig. 2). These genes are part of the ARE-gene battery and are involved in processes such as xenobiotic detoxification (e.g., NADPH oxidoreductase [NQO1]), reactive oxygen species detoxification (e.g., SOD1), heme detoxification (e.g., heme oxygenase), and glutathione synthesis (e.g., γ-glutamyl cysteine ligase light chain). Furthermore, human aldo-keto reductase (AKR) genes AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, AKR7A2, and AKR7A3 are regulated by the Keap1/Nrf2 system [77]. AKR1C2, also known as 3,3α-hydroxysteroid hydrogenase, primarily facilitates NADPH-dependent reactions. Among others, it catalyzes the conversion of dihydrotestosterone into its inactive form, 3α-androstanediol. Furthermore, it is involved into the transformation of

dihydropregnanolone into allopregnanolone. These processes are accomplished through the enzyme's catalytic activity in conjunction with the cofactor NADPH.

Allopregnanolone is an allosteric effector of gamma-aminobutyric acid (GABA) alpha receptors. Depending on the environment, dihydrotestosterone appears to have both growth-promoting and protective effects on cancer cells, with particular significance in breast and prostate malignancies [78]. Inhibitors already exist for both reaction pathways, which could be used to develop an improved therapy [77,79-83]. Furthermore, AKR1C2 utilizes the prostaglandins PGD2 and PGE2 as its substrates. Both resulting products interact with the prostaglandin F receptor, which then activates NF-κB nuclear factor 'kappa light chain-enhancer of activated B-cells (NF-κB) via the mitogen activated protein (MAP) kinase signaling pathway and thus leads to inflammation and proliferation [84].

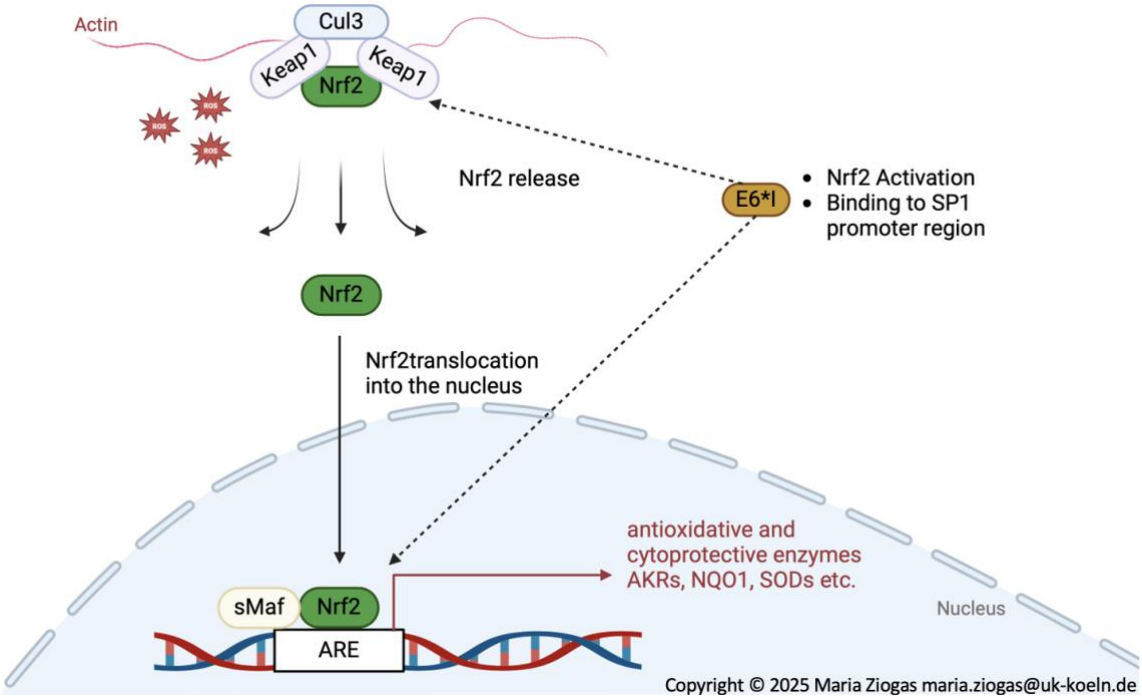

Fig. 2: Nrf2-Keap1-CUL3 pathway and E6*I interaction

Under normal conditions, Nrf2 binds to Keap1 and Cullin 3, preventing its activity. Upon release, Nrf2 translocates into the nucleus, partners with sMaf, and binds antioxidant response elements (ARE) to induce cytoprotective genes (e.g., AKRs, NQO1, and SODs). E6*I promotes Nrf2 activation partially via the SP1 promoter region, thereby enhancing the transcription of AKRs.

2.3.2. Aldo-keto reductases and OPSCC

AKR1C1, AKR1C2, and AKR1C3 play a role in lipid metabolism, including the processing of steroid hormones, and function as phase I detoxification enzymes, allowing them to metabolize various exogenous substances [77]. For instance, AKR1C2 influences carcinogenesis and prognosis by converting nicotine-derived nitrosaminoketone (NNK), a potent carcinogen found in tobacco, into its less harmful form, nitrosamine alcohol (NNAL) [85]. Additionally, the

upregulation of AKR1C2 and related enzymes helps to prevent the formation of ROS and ROS-derived peroxides, such as toxic lipid oxides [79].

Cisplatin, commonly used in adjuvant therapy and for treating non-operable OPSCC, is believed to induce cytotoxic lipids such as 4-hydroxynonenal. Stress-induced upregulation of AKR1Cs in cells may hinder the accumulation of these cytotoxic lipids in tumor cells, reducing treatment efficacy [79]. Moreover, AKR1C2 acts as a type of 3-hydroxy-steroid dehydrogenase that converts steroid hormones, such as progesterone, testosterone derivatives, and estrogen [84,86].

Interestingly, AKR1Cs create feedback loops that enhance their own expression by regulating Nrf2. For example, the conversion of estrone to 17β-estradiol by AKR1Cs stimulates Nrf2 expression and induces the activation of estrogen-related receptors, including estrogen-related receptor α (ERRα) [87,88].

AKR1C2 influences overall survival by interacting with various metabolic pathways and functions as an oncogene by activating the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway [89,90]. This activation inhibits apoptosis and promotes cell proliferation. Additionally, many head and neck squamous cell carcinomas (HNSCCs) harbor mutations in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene, which enhances signaling through the PI3K pathway and stimulates tumor cell growth [91,92]. PI3K signaling also triggers the production of cyclooxygenase-2 (COX-2) through the action of immunosuppressive PGE2 [84]. AKR1C2 plays a role in prostaglandin metabolism by promoting proinflammatory and proliferative prostaglandin F variants, while inhibiting apoptosis-inducing PGJ2 [79]. As most HPV infections are asymptomatic and resolve on their own, it has been hypothesized that endogenous or exogenous stressors are necessary for the virus to persist and potentially lead to malignant transformation. Synergistic interactions between HR-HPV infection and oxidative stress have been suggested as potential trigger factors. For instance, chronic inflammation caused by persistent HPV infection leads to a sustained increase in ROS levels, while reducing the body's antioxidant capacity [93]. Recent studies have suggested that oxidative stress may play a key role in promoting persistent HPV infection and facilitating integration of the viral DNA into the host genome [94]. Furthermore, the viral splice product E6*I of the HPV16-E6 protein increases AKR1C1 and AKR1C3 expression by binding to their promoter regions[95] and induces the oxidative stress cascade by activating the Nrf2-Keap1-CUL3 pathway [96].

2.3.3. Oxidative stress and HPV 16 splice-product E6* I

Sequence analyses of the splice donor and splice acceptor sites in the E6 reading frame and in vitro studies showed that truncated variants are formed in addition to the full-length E6 protein. Most data are available for E6*I, which is translated from alternatively spliced mRNA

using intron I. The amino-terminal E6*I domain of 44 amino acids corresponds to that of the full-length E6 protein; it is linked to a carboxy-terminal section of approximately 3 amino acids coded by 9 nucleotides. Transcripts encoding an E6*I protein have been detected in almost all alpha-papilloma viruses [97-99] (Fig. 3).

E6*I has anti-apoptotic effects and may regulate the expression of E6 [100]. In addition, E6*I can induce oxidative stress and DNA damage, which are critical for the viral life cycle[101]. HPV16-E6*I can directly promote AKR1C1 and AKR1C3 expression by binding to the specificity protein 1 (SP1) binding sites in their promoter regions [69,102]. Furthermore, Wanichwatanadecha et al. demonstrated that E6*I transfection in an HPV-negative cervical carcinoma cell line (C33A) led to the upregulation of AKR1C1 and AKR1C3 [95]. This suggests a direct promoter effect of E6*I on these enzymes via SP1-binding sites and Nrf2 activation [103,104]. Therefore, its expression in persistently infected cells could predispose them to genome damage and integration [105].

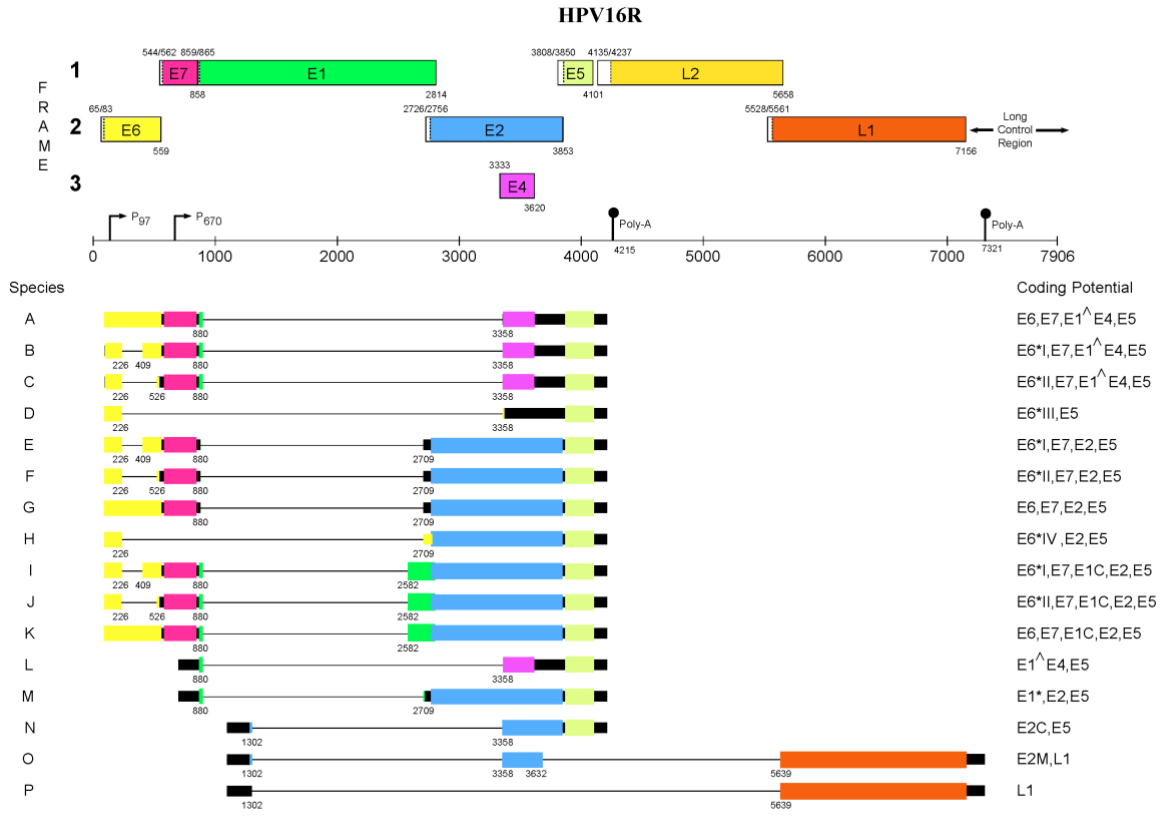

Fig. 3: Spliced isoforms of the HPV E6 and E7 oncogene [106].

2.4. Research questions and aim of the work

The present study focusses on analyzing AKR1C2 expression in HPV-positive and HPV-negative OPSCC. The enzyme AKR1C2, which belongs to the aldo-keto reductase family, plays a role in metabolizing steroid hormones and neutralizing harmful lipids produced by ROS. This enzyme is influenced by various factors, including oxidative stress, which upregulates it through the Nrf2/Keap1 signaling pathway. Under unstressed conditions, Keap1 binds Nrf2, resulting in its degradation, but oxidative stress releases Nrf2, allowing it to activate AKR gene transcription. Previous studies conducted by our research group revealed that increased expression of AKR1C1 and AKR1C3, genes associated with oxidative stress, is linked to unfavorable outcomes in OPSCC. This correlation may be affected by HPV proteins, such as E6*I, in HPV-positive cancers or by mutations in the Nrf2/Keap1 pathway in HPV-negative cases.

Furthermore, AKR1C2's role in tobacco carcinogen metabolism may link it to HPV-negative tumor development. The immunohistochemical analysis performed in this study examined AKR1C2 expression and HPV status in tumor samples from 51 OPSCC patients. Furthermore, the HPV16 E6*I splice variant as well as full-length E6, respectively, were overexpressed by transfecting expression vector constructs in $p53^{WT}$ Human Embryonic Kidney 293 (HEK293) cells to assess their impact on AKR enzyme expression to prove whether E6*I may regulate AKR1C2 expression in parallel to AKR1C1 and AKR1C3. This study aimed to enhance our understanding of the role of AKR1C2 in OPSCC, particularly in relation to HPV infection and oxidative stress. The insights gained from these findings might be incorporated into personalized treatment strategies and may offer insights into the mechanisms underlying gender-specific differences in OPSCC outcomes. Future studies should extend these findings by investigating additional biomarkers and potential therapeutic targets.

3. Published original article

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Article

Analysis of Expression and Regulation of AKR1C2 in HPV-Positive and -Negative Oropharyngeal Squamous Cell Carcinoma

Maria Ziogas [1,2,*], Oliver Siefer [1,2], Nora Wuerdemann [3], Harini Balaji [1,2], Elena Gross [4], Uta Drebber [5], Jens Peter Klussmann [1,2] and Christian U. Huebbers [1,2]

[1] Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital of Cologne, 50937 Cologne, Germany; oliver.siefer@uni-koeln.de (O.S.); harini.balaji@uk-koeln.de (H.B.); jens.klussmann@uk-koeln.de (J.P.K.); christian.huebbers@uk-koeln.de (C.U.H.)
[2] Molecular Head and Neck Oncology, Translational Research in Infectious Diseases and Oncology (TRIO) Research Building, University Hospital of Cologne, 50937 Cologne, Germany
[3] Department of Internal Medicine, Faculty of Medicine, Center for Integrated Oncology Aachen Bonn Cologne Duesseldorf, University Hospital Cologne, 50937 Cologne, Germany; nora.wuerdemann@uk-koeln.de
[4] Department of Neurology, University Hospital of Cologne, 50924 Cologne, Germany; elena.gross@uk-koeln.de
[5] Institute for Pathology, University Hospital of Cologne, 50937 Cologne, Germany; uta.drebber@uk-koeln.de
* Correspondence: maria.ziogas@uk-koeln.de

Simple Summary: Oropharyngeal Squamous Cell Carcinoma (OPSCC) represents a significant fraction of head and neck cancers, with a challenging five-year survival rate of only 50%. Key risk factors include tobacco and alcohol consumption and infection with human papillomavirus (HPV), particularly HPV16. Distinct biological differences exist between HPV-positive and HPV-negative OPSCC, including differences in mutation patterns and gene expression profiles. This study focuses on aldo-keto reductases (AKRs), specifically AKR1C2, which are involved in cellular stress management and detoxification processes, particularly in cisplatin-resistant tumors. This study investigates the role of AKR1C2 in HPV-positive OPSCC and its effect on patient outcomes. The findings indicate that increased levels of AKR1C2 are linked to unfavorable prognosis, particularly in male patients, while higher levels in female patients indicate a favorable prognosis.

Abstract: Head and Neck Squamous Cell Carcinoma (HNSCC), particularly Oropharyngeal Squamous Cell Carcinoma (OPSCC), is a major global health challenge due to its increasing incidence and high mortality rate. This study investigates the role of aldo-keto reductase 1C2 (AKR1C2) in OPSCC, focusing on its expression, correlation with Human Papillomavirus (HPV) status, oxidative stress status, and clinical outcomes, with an emphasis on sex-specific differences. We analyzed AKR1C2 expression using immunohistochemistry in formalin-fixed, paraffin-embedded tissue samples from 51 OPSCC patients. Additionally, we performed RT-qPCR in cultured HPV16-E6*I and HPV16-E6 overexpressing HEK293 cell lines ($p53^{WT}$). Statistical analyses were performed to assess the correlation between AKR1C2 expression and patient data. Our results indicate a significant association between increased AKR1C2 expression and higher AJCC classification ($p = 0.009$) as well as positive HPV status ($p = 0.008$). Prognostic implications of AKR1C2 varied by sex, whereby female patients with high AKR1C2 expression had better overall survival, whereas male patients exhibited poorer outcomes. Additionally, AKR1C2 expression was linked to HPV status, suggesting a potential HPV-specific regulatory mechanism. These findings underscore the complex interplay among AKR1C2, HPV, and patient sex, highlighting the need for personalized treatment strategies for OPSCC. Targeted inhibition of AKR1C2, considering sex-specific differences, may enhance therapeutic outcomes. Future research should investigate these mechanisms to enhance treatment efficacy.

Keywords: human papillomavirus; oropharyngeal squamous cell carcinoma; aldo-keto-reductase 1 C2; oxidative stress

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1. Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is the sixth most common malignancy worldwide, with an estimated 878,000 new cases reported in 2022 [1]. Squamous Cell Carcinoma of Oropharyngeal Origin (OPSCC) is a subgroup of HNSCC. Although treatment has improved in recent years, the global mortality rate remains high. Approximately 50% of patients with OPSCC survive the first five years after diagnosis [2,3].

The most common risk factors for the development of HNSCC, in general, are tobacco smoke, often in combination with excessive alcohol abuse [4], and infections with high-risk human papillomavirus (HPV) genotypes, with HPV16 being particularly associated with OPSCC [5]. The Global Cancer Observatory (GLOBOCAN) predicted an even greater increase in incidence, with an estimated rate of 30%. This effectively means that we would be faced with 1.08 million new cases per year by 2030 [6,7]. Data from the United States show that the incidence of HPV-associated OPSCC already surpassed that of HPV-positive cervical cancer [8–10].

Since HPV-positive and HPV-negative OPSCC show different clinicopathological characteristics, as well as biological profiles, mutation patterns, and expression signatures, the TNM Classification of OPSSC has been adapted accordingly to distinguish between these two groups. Using the surrogate marker $p16^{INK4a}$, HPV-related ($p16^{INK4a}$-positive) and HPV-negative ($p16^{INK4a}$-negative) OPSCC can be differentiated, thus, different prognoses can be considered [11]. However, the benefits of different treatment strategies in patients with HPV-associated and HPV-non-associated OPSCC are still being discussed [12]. A subpopulation of 20–25% of HPV-positive OPSCC patients present with poor prognosis due to locoregional recurrence or metastatic disease, which may be linked to additional risk factors such as smoking, EGFR overexpression, advanced nodal stage, and chromosomal instability [13–16]. Strikingly, the risk for women developing HPV-positive OPSCC is approximately four times lower than for men [17]. A possible explanation for this apparent discrepancy could be the different hormonal signals between men and women, which have been discussed as cofactors for HPV-related cancers [18].

This study analyzes the role of aldo-keto reductases (AKR), with a particular focus on AKR1C2. AKR1Cs are important in the epithelial response to oxidative stress. Together with other associated proteins, such as its family members AKR1C1, AKR1C3, NADPH oxidoreductase (quinone 1) (NQO1), superoxide dismutase (SOD1), and haem oxygenase (HQ), it belongs to the group of genes controlled by antioxidant response elements (ARE), which are increasingly expressed in the case of electrophilic or oxidative stress. The expression of ARE element-containing genes is coupled to the Nrf2-KEAP1-CUL3 pathway and therefore predominantly dependent on the regulatory function of these upstream proteins [19]. We previously showed that the upregulation of the ARE element-induced genes AKR1C1 and AKR1C3 correlates with poor prognosis in patients with oropharyngeal carcinomas and is associated with the oxidative stress response system [20].

AKR1C1, AKR1C2, and AKR1C3 metabolize lipids, including steroid hormones, and serve as phase I detoxification enzymes, enabling them to metabolize exogenous substrates [19]. For example, AKR1C2 affects carcinogenesis and prognosis by reducing one of the strongest nitrosamine carcinogens in tobacco, nicotine-derived nitrosaminoketone (NNK), into its detoxified substrate, nitrosamine alcohol (NNAL) [21]. Furthermore, the upregulation of AKR1C2 and its family members prevents the accumulation of reactive oxygen species (ROS) and ROS-derived peroxides, such as cytotoxic lipid oxides [22]. Cisplatin is typically used in adjuvant therapy and in the treatment of non-operable OPSCC. It has been proposed that cisplatin induces cytotoxic lipids such as 4-hydroxynonenale (4-HNE), and cellular stress-induced upregulation of AKR1Cs might prevent their intended accumulation in tumor cells [22]. Furthermore, AKR1C2 is a type 3 hydroxysteroid dehydrogenase that transforms steroid hormones such as progesterone, testosterone derivates, and estrogen [23,24]. Interestingly, AKR1Cs generate feedback loops, amplifying their own expression by controlling NRF2 expression. For example, the conversion of estradione (E1) to 17β-estradiol (E2) by AKR1Cs promotes NRF2 expression and results in the induction of

estrogen receptors such as the estrogen-related receptor α (ERRα) [25,26]. The involvement of sex hormone levels and metabolism and the resulting sex- and patient-specific differences may be underlined by the common observation that women develop OPSCC less frequently. Moreover, based on the clinical data of 1629 OPSCC patients, we have recently reported that women present with significantly longer overall survival than men [27].

In the present study, we aim to investigate the correlation between HPV infection, AKR1C2 expression, and oxidative stress mechanisms in relation to clinical data, such as sex, TNM classification, and survival in OPSCC patients. The viral splice product E6*I of the HPV16-E6 protein increases AKR1C1 and C3 expression by binding to their promoter regions [28]. Although AKR1C2 has many similarities to AKR1C1 and AKR1C3, its expression is regulated by independent mechanisms. In addition, AKR1C2 has specific enzyme characteristics, and not all its functions are yet known.

2. Materials and Methods

2.1. Subjects and Materials

Formalin-fixed, paraffin-embedded (FFPE) tissue samples from 51 Oropharyngeal Squamous Cell Carcinomas of patients treated at the Department of Otorhinolaryngology and Head and Neck Surgery of the University Hospital of Cologne, Germany, between 2004 and 2011 were analyzed. HPV status was determined using routine PCR and $p16^{INK4a}$ immunohistochemical staining. In total. 25 (49%) samples were HPV-negative and 26 (51%) were HPV-positive (Table 1). AKR1C1, AKR1C3, and NRF2 expression levels were determined in previous studies based on the same cohort and were included in this analysis [20].

Table 1. Summary of clinicopathological features of patients analyzed in this study.

		Total (1)		AKR1C2 Tumor Staining				
				$AKR1C2^{HIGH}$ (2)		$AKR1C2^{LOW}$ (2)		
Clinicopathological Feature		*n*	%	*n*	%	*n*	%	χ^2
Mean age (years)		51		55.125		60.162		
Sex								
	Male	39	76.5	21	42.9	16	32.7	
	Female	12	23.5	8	16.3	4	8.2	0.738
T classification								
	pT1 and pT2	23	45.1	10	20.4	12	24.5	
	pT3 and pT4	28	54.9	19	38.8	8	16.3	0.090
N classification								
	pN0	13	25.5	6	12.2	7	14.3	
	pN1–2 (3)	39	74.5	23	46.9	13	26.5	0.331
M classification								
	pM0	49	96.1	28	57.1	19	38.8	
	pM1	2	3.9	1	2.0	1	2.0	1.000
AJCC classification								
	I	14	27.5	5	10.2	8	16.3	
	II	12	23.5	4	8.2	8	16.3	
	III	10	19.6	8	16.3	2	4.1	
	IV	15	29.4	12	24.5	2	4.1	**0.009**
Relapse								
	Yes	23	45.1	13	26.5	9	18.4	
	No	28	54.9	16	32.7	11	22.4	1.000
Death								
	Yes	23	47.0	13	26.5	6	12.2	
	No	26	53.3	16	32.7	14	28.6	0.377
HPV-status								
	Negative	25	49.0	19	38.8	5	10.2	
	Positive	26	51.0	10	20.4	15	30.6	**0.008**
Smoking								
	Yes	40	78.4	22	45.8	16	33.3	
	No	11	21.6	6	12.5	4	8.3	1.000

Table 1. *Cont.*

		Total (1)		AKR1C2 Tumor Staining AKR1C2^{HIGH} (2)		AKR1C2^{LOW} (2)		
Clinicopathological Feature		*n*	%	*n*	%	*n*	%	χ^2
Alcohol								
	Yes	25	49	14	28.6	11	22.4	
	No	26	47	15	30.6	9	18.4	0.773
Localization								
	Tonsil	31	60.8	16	32.7	15	30.6	
	Tongue base	15	29.4	8	16.3	5	10.2	
	Soft palate	5	9.8	5	10.2	0	0	0.122
NRF2 expression								
	Nuclear	14	27.5	8	16.3	6	12.2	
	Cytoplasmic	37	72.5	21	42.9	14	28.6	1.000
AKR1C1 expression								
	AKR1C1 (+)	15	29.4	20	40.8	6	12.2	
	AKR1C1 (−)	36	70.6	9	18.4	14	28.6	1.000
AKR1C3 expression								
	AKR1C3 (+)	15	29.4	20	40.8	6	12.2	
	AKR1C3 (−)	36	70.6	9	18.4	14	28.6	1.000

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. (2) Relative staining compared to normal epithelium. AKR1C2^{HIGH} means higher expression in tumor cells compared to normal epithelium, and AKR1C2^{LOW} means less or equal staining in tumor cells compared to normal epithelium. χ^2: Chi-Square test for significance. For mean age, ANOVA is used to measure significance. Significant values are highlighted in bold.

2.2. 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 was obtained from all patients.

2.3. Immunochemistry

Immunohistochemical staining was performed on 4 µm thick FFPE tissue sections according to routine protocols using indirect immunolabelling with DAB detection. AKR1C2 expression was detected using rabbit polyclonal antibodies (catalogue number PA5-36572, 1:200 in PBS; Thermo Fisher Scientific, Darmstadt, Germany).

Briefly, sections were deparaffinized with Roti®-Histol (Carl Roth, Karlsruhe, Germany) and rehydrated using a descending alcohol series. Subsequently, the sections were incubated overnight at 70 °C in 0.01 M citrate buffer (pH 6.0) for antigen retrieval, followed by incubation with AKR1C2 antibody at 4 °C overnight. After washing and incubation with corresponding biotinylated goat anti-rabbit secondary antibodies (Vector, Burlingame, CA, USA; 1:250), slides were incubated with avidi-biotin-peroxidase complex (ABC; Vectastatin ABC kit, Vector), and the peroxidase activity was developed with 0.05% 3,3′-diaminobezidine tetrahydrochloride (DAB, Vector) in 0.05 M Tris-HCl (pH 7.6). Sections were mounted in Entellan (Merck, Darmstadt, Germany).

Controls included tumor-free human control tissues selected based on the human protein atlas showing no, moderate, and strong expression, respectively (liver, cervix, and tonsils) [29]. Staining without primary antibodies and IgG isotype controls was negative in all tissues.

Data on the protein expression of AKR1C1, AKR1C3, and NRF2 were previously published for the same cohort and were included for statistical comparison [20].

2.4. Cell Culture and Transfection

HEK293 cells (ATCC: CRL-1573) were grown in DMEM high-glucose medium supplemented with 10% FBS (both Thermo Fisher Scientific, Darmstadt, Germany) under standard conditions (humidified incubator at 37 °C, 5% CO_2). HPV16-E6 and HPV16-E6*I were cloned from cDNA into pEGFP-N1 vector (Takara Clontech, Saint-Germain-en-Laye, France). Cells were transfected with resulting HPV16-E6*I-GFP, HPV16-E6-GFP, and GFP control vector constructs, respectively, using lipofectamine according to the instructions of the manufacturer (Thermo Fisher Scientific, Germany). Stable HEK293 clones were obtained by selection with 1.2 mg/mL G418.

2.5. RT-qPCR Expression Analysis

RT-qPCR was performed as described previously [20]. In brief, RNA was extracted from cultured cell lines using the Qiagen RNeasy mini kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). In total, 500 ng of total RNA was reverse transcribed (iScript cDNA synthesis kit, BioRad Laboratories, Munich, Germany) and qPCR was performed using iTaq SYBR Green Supermix (BioRad). Amplification was performed using previously described primers applying standard protocols [20]. Hypoxanthine Phosphoribosyltransferase (HPRT) was used for the normalization of mRNA levels.

2.6. Statistics

Clinicopathological features were analyzed using cross-tabulations, the χ^2 test, and Fisher's exact probability test with SPSS Statistics for Mac version 28.0.1.0 (IBM Software, Armonk, NY, USA). Overall survival rates were estimated over a 5-year period using the Kaplan–Meier algorithm for incomplete observations. Overall survival describes the interval between the date of initial diagnosis and the last date on which the vital status was recorded as "alive" (censored) or the date of death (uncensored). Univariate analyses of variables were performed using the log-rank (Mantel-Cox) test. The minimum sample size for subgroup analysis was determined prior to analysis with a power of 90% and a significance level of 0.05 with 8 samples for each group.

Data analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). The significance level was set at $p < 0.05$ for all calculations.

3. Results

3.1. Immunohistochemical Detection of AKR1C2

Immunohistochemistry was used to determine the expression of AKR1C2 in epithelial tissues and tumors. In non-tumor tissue, lymphocytes were negative and squamous epithelial keratinocytes showed predominantly no or weak nuclear staining, whereas muscle cells and endothelial cells exhibited strong staining. Control staining without primary antibodies and IgG isotype controls was negative in all tissues The cohort comprised 51 patients, of which 49 FFPE samples with sufficient material were available. Consistent with our previously observed expression pattern for AKR1C1 and AKR1C3 [20,30], staining against AKR1C2 was also positive in adjacent non-tumorous squamous epithelia. As such, we decided to evaluate the staining intensity of both the tumor and the adjacent epithelium and relate them to each other in further analysis, as previously described for AKR1C1/C3 (Figure 1) [20].

The resulting protein intensity ratios showed 29 OPSCC (59.2%) with stronger staining in the tumor compared to the adjacent epithelium ($AKR1C2^{HIGH}$), and the remaining 20 OPSCC (40.8%) showed lower staining than the adjacent epithelium ($AKR1C2^{LOW}$) (Table 1). Moreover, 25 OPSCC were HPV negative (49%), of which 5 presented $AKR1C2^{LOW}$ (10.2%), whereas 15 were $AKR1C2^{HIGH}$ (30.6%). In the case of the 26 HPV-positive OPSCC (51%), 15 presented $AKR1C2^{LOW}$ (30.6%) and 10 $AKR1C2^{HIGH}$ (20.4%) ($\chi^2 = 0.008$). Furthermore, increased AKR1C2 expression correlated with higher AJCC classification ($\chi^2 = 0.009$). No significant correlation was found for any of the other parameters analyzed, including alcohol and tobacco consumption and sex.

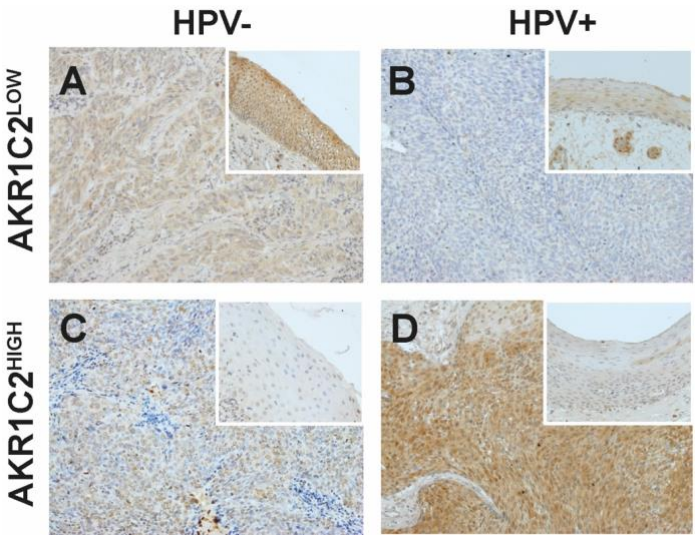

Figure 1. Representative immunohistochemical staining against AKR1C2 in non-tumorous (small rectangular images) and tumor tissue samples (**A–D**). AKR1C2^{LOW} indicates lower and AKR1C2^{HIGH} indicates higher expression of AKR1C2 compared to the adjacent non-tumorous epithelium. (**A**,**C**) HPV-negative (HPV−) OPSCC, (**B**,**D**) HPV-positive (HPV+) OPSCC. V = x200.

3.2. AKR1C2 Protein Expression, HPV, and Survival in OPSCC

AKR1C2 protein expression was correlated with survival outcomes in combination with clinicopathological data such as sex, HPV status, tumor status, smoking history, alcohol consumption, and protein expression levels of AKR1C1, AKR1C3, and NRF2 (Table 1). Whereas AKR1C2 expression in general (Hazard Ratio (HR) 0.4953, 95% Confidence Interval (CI) 0.1899–1.209, p = 0.1229) and in combination with T-Status (HR = 2.734, 95% CI 0.960–7.790, p = 0.3162), N-Status (HR 1.785, 95% CI 0.920–3.464, p = 0.5722), smoking habit (HR 1.488, 95% CI 0.433–5.116, p = 0.7219), and drinking habits (HR 1.530, 95% CI 0.942–2.483, p = 0.1988) did not correlate with significant outcomes, AKR1C2 expression presented with a trend for significant correlation with HPV status (HR 1.818, 95% CI 0.669–4.938 p = 0.2129; log-rank trend test p = 0.0368). However, when considering patient sex, OS was significantly different (HR 1.235, 95% CI 0.704–2.167, p = 0.0151). Remarkably, female sex combined with AKR1C2 positivity was predictive of a more favorable outcome, while low AKR1C2-expressing tumors in males were correlated with a better outcome. Therefore, we performed subgroup analyses for both sexes, considering both AKR1C2 expression levels and HPV status into account. Whereas, in women, AKR1C2^{HIGH} tumors presented with a tendency for beneficial survival regardless of HPV status (HR = 0.333, 95% CI 0.028–3.977, p = 0.0350), HPV+/AKR1C2^{LOW} tumors presented with far better survival probability, followed by intermediate outcomes for HPV+/AKR1C2^{HIGH} and HPV−/AKR1C2^{LOW} tumors (HR 2.300, 95% CI 0.734–7.201, p = 0.0168). HPV−/AKR1C2^{HIGH} tumors presented with the most unfavorable outcome.

For death within 5 years, as well as higher tumor size, a significant correlation was observed. Of note, HPV status appeared to have a strong correlation with AKR1C2 protein expression (p = 0.022) (Figure 2).

In general, the AKR1C2^{HIGH} group of patients was affected by death earlier than the AKR1C2^{LOW} group. Here, low AKR1C2 tumor staining was correlated with an overall survival of 70–80% by the end of five years. In addition, women in this cohort showed

better overall survival when AKR1C2 expression in tumor tissue was higher than that in men with high AKR1C2 expression.

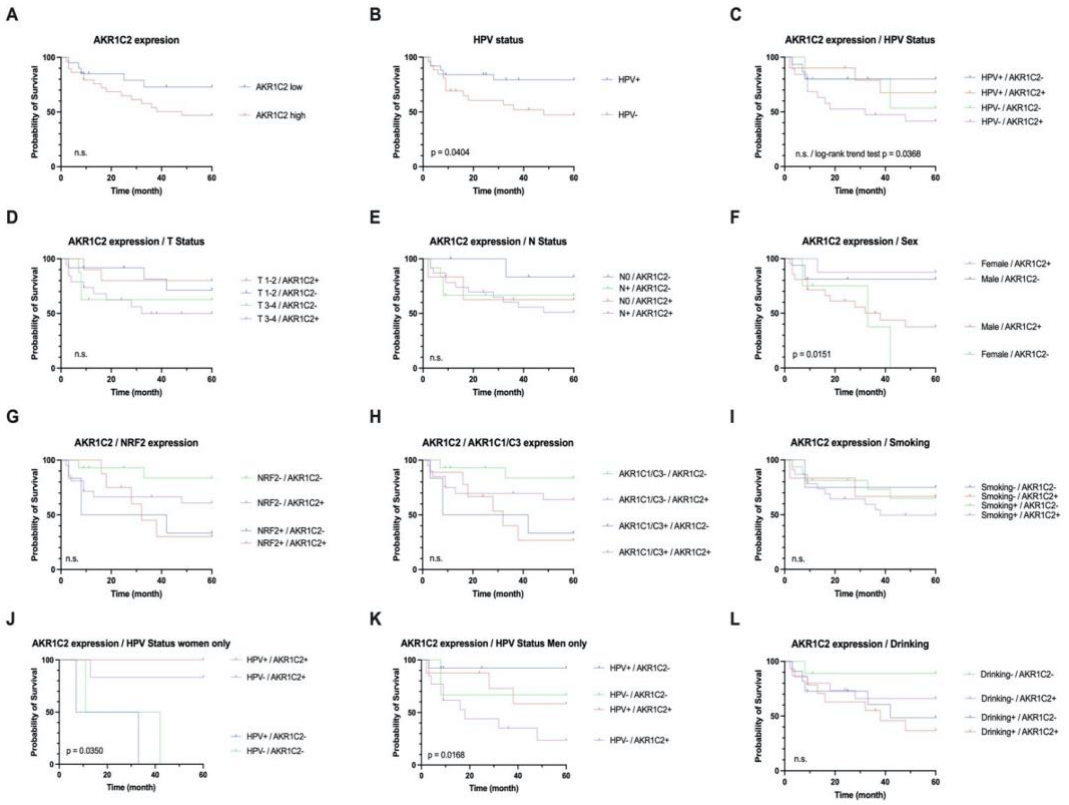

Figure 2. Univariate survival analysis for AKR1C2 expression status with low vs. high protein expression in tumor compared to adjacent non-tumorous epithelium. *p* value was derived by log-rank/Mantel–Cox test. Analyses of HPV status (**B**), AKR1C2 expression combined with sex, and combinations of AKR1C2 expression and HPV status in women (**J**) and men (**K**) proved to be significant.

3.3. Correlation of AKR1C2 with AKR1C1 and AKR1C3 and NRF2

Considering the expression levels of AKR1C1, AKR1C3, and NRF2, AKR1C2 was found to be an independent predictive factor (AKR1C1 and AKR1C3 HR 1.710, 95% CI 1.084–2.698, $p = 0.0575$; NRF2 HR 0.413, 95% CI, 0.166–1.026, $p = 0.1096$) (Table 1).

*3.4. Effects of HPV16-E6*I on AKR1C2 mRNA Expression*

Stable HPV16-E6- or HPV16-E6*I-overexpressing HEK293 cells ($p53^{WT}$) were analyzed by RT-qPCR for the expression of AKR1C2 and its counterparts AKR1C1 and AKR1C3, respectively. While E6*I- but not E6-overexpressing HEK293 cells showed increased AKR1C1 and AKR1C3 expression, AKR1C2 expression was not affected by E6*I or E6, respectively (Figure 3).

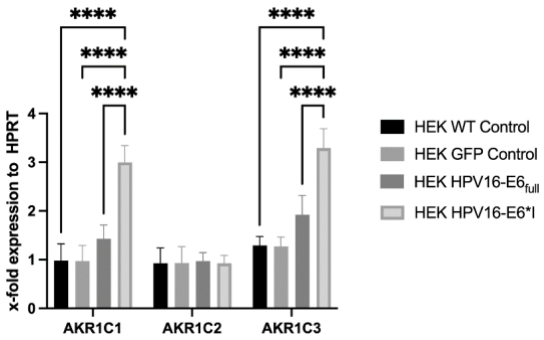

Figure 3. mRNA expression of AKR1C1, AKR1C2, and AKR1C3 in HEK293 cells overexpressing HPV16-E6 or HPV16-E6*I. Hypoxanthine Phosphoribosyltransferase (HPRT) was used for normalization of mRNA levels. (****) $p < 0.00001$.

4. Discussion

The human aldo-keto reductase family has recently emerged as a promising marker in various cancers and is a key factor in the development of resistance to radio- and chemotherapy [22]. Resistance mechanisms are based either on direct metabolic involvement or contribution to the elimination of cellular stress (e.g., from reactive oxygen species and lipid peroxides). Of particular interest is the possibility of pharmacologically inhibiting AKR1Cs with easily administered and well-known substances, such as NSAID derivatives, thus preventing therapeutically unfavorable protective mechanisms against cellular stress.

In a previous study, we observed the upregulation of AKR1C1 and AKR1C3 expression in a subgroup of HPV16-positive OPSCC along with upregulated HPV16-E6*I mRNA expression. AKR1C1/C3 overexpression has also been associated with poor prognosis in both HPV-positive and HPV-negative OPSCC subgroups [20,28].

AKR1C2 is located in the same genomic region on chromosome 10p15-14 and contains all four aldo-keto reductase family 1 member C genes. The proteins show high sequence homology, namely AKR1C1/AKR1C2 with 98% homology, differing in only 7 amino acids, whereas AKR1C2/AKR1C3 show 87% homology and differ in 43 amino acids [31]. However, AKR1Cs have independent substrate specificities, implying an independent (patho-)physiological role. Furthermore, there is evidence that AKR1C2 expression is sex-dependent in several tissues, underlined by its involvement in progesterone and dihydrotestosterone metabolism [32–34]. Therefore, the impairment of sex-dependent turnover of these compounds might result in subsequent consequences for diseased tissue.

Although all other AKR1Cs are encoded on the forward strand, AKR1C2 is encoded in the opposite direction. However, this means that AKR1C2 shares gene regulatory elements with its neighbors, as shown for a cis-regulatory region common to AKR1C2 and AKR1C1, which raises the possibility of joint regulation [35].

The alternatively spliced version of the HPV16-E6 full-length protein (HPV16-E6*I) can directly promote AKR1C1 and AKR1C3 expression by binding to SP1 binding sites in their promoter regions [20,28]. Furthermore, E6*I promotes signaling pathways of the oxidative stress response, including the activation of NRF2 signaling [36,37]. Our observation that AKR1C2 is upregulated in a subgroup of HPV-positive OPSCC patients and is a strong indicator of prognosis suggests HPV-specific regulation. However, the overexpression of HPV16-E6*I did not alter AKR1C2 mRNA expression, as observed for AKR1C1 and AKR1C3, indicating an alternative HPV-induced regulation. Furthermore, AKR1C2 shows independent protein expression compared to its counterparts AKR1C1 and AKR1C3 [20]. AKR1C4 expression was not included, as it is reported to be exclusively expressed in a liver-specific manner and was negative in our previous analyses [20]. The AKR1C family enzymes including AKR1C2 are capable of detoxifying components of tobacco smoke such

as nicotine-derived nitrosamine ketones (NKKs). Cells can protect themselves against external stressors by increasing enzyme expression. Additionally, it has been shown that AKR1C2 can also reduce chemotherapeutics such as cisplatin, which leads to cisplatin-resistant tumors [38]. However, a history of alcohol and/or tobacco consumption did not affect AKR1C2 expression levels or specific prognoses.

AKR1C2 has been reported to not only affect overall survival by interacting with various metabolic pathways but also to act as an oncogene by activating the PI3K/AKT pathway [38,39], thereby inhibiting apoptosis and increasing proliferation. Furthermore, several HNSCCs carry cancer-associated mutations in the PIK3CA gene, which promotes signaling via the PI3K pathway and thus stimulates tumor cell growth [40,41]. PI3K signaling induces the production of cyclooxygenase-2 (COX-2) via immunosuppressive prostaglandin E2 (PGE2) [24]. AKR1C2, in turn, is involved in prostaglandin metabolism by favoring proinflammatory and proliferation-promoting prostaglandin F variants, thus inhibiting the apoptosis-promoting prostaglandin J2 [24].

Our observation that the subgroup of female patients presenting with increased AKR1C2 expression showed more favorable overall survival is consistent with recent findings of significantly better 5-year OS in women with HNSCC and in the OPSCC subgroup [27,42]. Different lifestyles, HPV status, immune responses, and hormonal influences were discussed as possible factors for these findings [43].

In regard to AKR1C1 and AKR1C3, it has been reported that they play a role in the metabolism of estrogen. This results in a feedback loop where estrogen increases NRF2 activity leading to increased AKR1C expression [25]. However, our findings indicate that AKR1C2 expression does not show a correlation with NRF2 expression, which suggests that it is not involved in this regulatory feedback loop.

However, E6-mediated repression of proliferator-activated receptor gamma co-activator 1α/estrogen-related receptor α (PGC-1α/ERRα) may contribute to the observed differences in AKR1C2 expression between sexes. Inactivating the PGC-1α/ERRα pathway results in a lower mitochondrial antioxidant capacity and, therefore, a reduced treatment resistance [26].

In recent years, interest in the pharmacological inhibition of AKR1Cs has increased because they catalyze key reactions in the metabolism of prostaglandins, steroidal hormones, and cytostatic substances, thus promoting the signaling pathways directly involved in oncogenesis. However, most of the available substances non-selectively inhibit all AKR1Cs. Important classes of such drugs are nonsteroidal anti-inflammatory drugs (NSAIDs), benzodiazepines, steroids, and flavonoids [44–47]. Interestingly, the most well-known NSAID, acetylsalicylic acid (aspirin), is known to exhibit potent inhibition of AKR1Cs [22]. Specific inhibitors of AKR1C1 (3-bromo-5-phenylsalicylic acid) and AKR1C3 (tolfenamic acid, indomethacin; phase I/II trial in prostate cancer, NCT02935205)) are also available. However, such a drug to inhibit AKR1C2 specifically is not available to date. However, the inhibition of AKR1C2 expression can be achieved by ursodeoxycholic acid (USDC), leading to a synergistic effect in cell lines when combined with cisplatin [38]. Nevertheless, some of these unspecific substances, including NSAIDs, are already therapeutically approved and therefore simply require a combination with well-known chemotherapeutic agents so they can be easily established clinically. The strategy of utilizing NSAIDs is supported by a study analyzing HNSCC with PIK3CA mutations or amplifications (which may implicate co-occurring AKR1C overexpression as already discussed) where regular NSAID use (≥6 months) markedly prolonged disease-specific survival [48,49] and the Nurses Health cohort study, in which the use of both aspirin- and non-aspirin-based NSAIDs prolonged the survival of ovarian cancer patients using the primary chemotherapeutic agent, cisplatin [50].

The present study is partly limited by the small number of cases included, particularly the number of female patients. Nevertheless, we obtained results comparable to those of previous studies with larger cohorts.

In conclusion, AKR1C2 expression in tumor tissue is sex-dependent and, therefore, has a different predictive value. Although increased expression in female patients is associated with a favorable prognosis, this is not the case for male patients. For this reason, future studies with (un)specific AKR1C inhibitors must consider the sex of patients.

5. Conclusions

This study provides an analysis of the expression and regulation of Aldo-Keto Reductase 1C2 (AKR1C2) in HPV-positive and HPV-negative oropharyngeal squamous cell carcinoma (OPSCC). The key findings indicate that increased AKR1C2 expression is significantly associated with positive HPV status and higher AJCC classification, reflecting its potential role in tumor progression. Notably, AKR1C2 expression exhibited a sex-specific prognostic impact, where high levels correlated with poorer outcomes in male patients but more favorable survival is suggested in female patients. This differential impact underscores the importance of considering sex-specific factors in OPSCC prognosis and treatment strategies. The implications of these findings are profound, particularly in the context of clinical and translational cancer research. The study highlights the need for personalized treatment approaches in OPSCC, potentially targeting AKR1C2, especially in HPV-positive cases. Given the association of AKR1C2 with oxidative stress mechanisms, the results also contribute to the broader understanding of how oxidative stress and detoxification pathways influence cancer development and progression. Despite these significant findings, the study has limitations that should be acknowledged. Further studies on larger cohorts with a higher proportion of female patients are needed to further substantiate the results presented here. Such studies may also demonstrate how an AKR1C2 evaluation can be integrated into routine pathological evaluation. Furthermore, additional research on AKR1C expression and inhibition by established pharmacological substances is warranted. In conclusion, this study emphasizes the critical role of AKR1C2 in the progression of OPSCC and its potential as a biomarker for tailoring personalized treatment strategies. The findings also highlight the complex interplay between viral infection, oxidative stress, and sex-specific factors in cancer biology, urging further exploration in these areas to enhance therapeutic efficacy and patient outcomes in head and neck cancers.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Medical Faculty of the University of Cologne, Germany (study number 11–346, 2011 for tumor tissue and 18–285, 2018 for keratinocytes).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available upon request from the corresponding authors. The data are not publicly available due to ethical restrictions.

Conflicts of Interest: All authors declare no conflicts of interest.

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4. Discussion

4.1. The role of oxidative stress in HPV-related OPSCC

Given that transient HPV infections are typically asymptomatic, it has been suggested that additional endogenous and exogenous stress factors may be required for malignant transformation and progression. Among the factors that promote migration and invasion, increased production of ROS, resulting in an elevated oxidative stress response, is discussed. In this metabolic state, lipids, saccharides, proteins, and nucleic acids undergo pathological modifications owing to oxidation reactions, and the accumulation of this damage has been linked to various cancers and other diseases [76]. This environment may also facilitate the unfavorable integration of HPV DNA into the host genome, for example, inefficient DNA repair mechanisms [101].

In an earlier study, we identified upregulation of AKR1C1 and AKR1C3 expression in a subset of HPV16-positive OPSCC patients, accompanied by increased levels of HPV16-E6*I mRNA. Overexpression of AKR1C1 and AKR1C3 has also been linked to unfavorable prognosis in both HPV-positive and HPV-negative OPSCC groups, highlighting their potential role as markers for disease progression [102,107].

AKR1C2 is located on chromosome 10p15-14 in a genomic segment that encompasses all four genes belonging to the aldo-keto reductase family 1C (AKR1C). Interestingly, while the other AKR1C genes are encoded on the forward strand, AKR1C2 is encoded on the reverse strand, sharing regulatory elements with neighboring genes, such as a cis-regulatory region common to AKR1C1 and AKR1C2, raising the possibility of coordinated regulation.

AKR1C2 shares significant sequence homology with AKR1C1 (98%, differing by only seven amino acids) and AKR1C3 (87%, with 43 amino acid differences). Despite these similarities, AKR1C enzymes exhibit distinct substrate specificities, indicating that they have independent physiological and pathological roles. AKR1C4 is not relevant to OPSCC tumor biology. It is exclusively expressed in the liver, and we did not observe any significant expression in OPSCC [102].

The HPV16-E6 oncoprotein is expressed in multiple isoforms. In vitro studies have demonstrated that the main splice variant, HPV16-E6*I, increases ROS levels, leading to higher oxidative stress and, consequently, more DNA damage. This increase in ROS is attributed to a decline in the cell's antioxidant capacity, as the presence of E6*I was found to reduce the expression of key antioxidant enzymes, including superoxide dismutase and glutathione peroxidase [96]. Additionally, HPV16-E6*I has been shown to activate Nrf2 signaling and directly stimulates AKR1C1 and AKR1C3 expression by binding to SP1 sites in their promoter regions [95,102,103,104]. In contrast, AKR1C2 mRNA expression was not similarly influenced by HPV16-E6*I, suggesting a distinct regulatory pathway for this gene in HPV-

positive OPSCC. Nevertheless, AKR1C2 expression was upregulated in a subgroup of HPV-positive OPSCC patients, suggesting that it may be a strong prognostic indicator. Its regulation appears to be HPV-specific but independent of AKR1C1 and AKR1C3 [108].

AKR1C2 exerts its influence on overall survival through multiple metabolic pathways and simultaneously acts as an oncogene by stimulating PI3K/AKT signaling [89,90]. The activation of this pathway results in enhanced tumor cell proliferation and reduced apoptotic activity. Mutations in the PIK3CA gene, which enhance PI3K signaling, are common in HNSCC and other squamous cell carcinoma [91,92]. The PI3K pathway stimulates the production of COX-2 via PGE2, an immunosuppressive agent. AKR1C2 is involved in the metabolism of prostaglandins and promotes the synthesis of prostaglandin F, which has pro-inflammatory effects and stimulates cell proliferation. Simultaneously, AKR1C2 suppresses PGJ2 production, which promotes apoptosis[84].

4.2. Sex differences in incidence and prognosis

In the cohort studied here, a subgroup of female patients with increased AKR1C2 expression demonstrated improved overall survival [108]. This finding is consistent with recent studies showing significantly better five-year overall survival rates among women with HNSCC, particularly in the OPSCC subgroup [13,109]. This survival advantage in women could be linked to hormonal influences, immune response variations, or different lifestyle factors such as lower alcohol and tobacco use [4,7].

Studies have shown that AKR1C1 and AKR1C3 are involved in estrogen metabolism, creating a feedback loop in which increased estrogen levels enhance Nrf2 activity, subsequently boosting AKR1C expression [86,110]. However, our data revealed that AKR1C2 expression did not correlate with Nrf2 levels, suggesting that it does not participate in this regulatory feedback loop.

Moreover, E6-mediated repression of proliferator-activated receptor gamma co-activator 1α/estrogen-related receptor α (PGC-1α/ERRα) may explain the differences observed in AKR1C2 expression between sexes, potentially reflecting the distinct ways in which estrogen hormones interact and modulate this pathway. Inactivation of the PGC-1α/ERRα pathway is associated with decreased mitochondrial antioxidant capacity, leading to diminished treatment resistance. This mechanism highlights the complex interplay between hormonal regulation and cancer metabolism [111,112].

Hormones play a crucial, often protective role in various types of cancers in women, including hepatocellular carcinoma. A case-control study conducted by Hashim et al. demonstrated an inverse correlation between the risk of developing HNSCC and endogenous as well as exogenous estrogen exposure. Specifically, research has revealed that females who delivered babies before turning 35 had a reduced likelihood of developing HNSCC compared to those who gave birth at an older age or had never been pregnant [113]. Additionally, female hormonal

pathways can be influenced by smoking and alcohol consumption. Smoking, in particular, is known to enhance estrogen catabolism, which may explain the differing effects of smoking on HNSCC risk between women and men [114].

Studies indicate that AKR1C2 exhibits sex-specific expression patterns across various tissues, which is attributed to its participation in progesterone and dihydrotestosterone metabolic processes [115-117]. This may lead to different effects on diseased tissues, depending on sex.

In contrast to the improved overall survival in the female subgroup with elevated AKR1C2 expression, males with high AKR1C2 expression had worse outcomes, particularly in the HPV-negative subgroup [108]. The sex-dependent role of AKR1C2 may be influenced by its involvement in the metabolism of steroid hormones, such as progesterone and dihydrotestosterone, which differ between men and women. These findings underscore the importance of considering sex as a factor in the prognosis and treatment of OPSCC and suggest that therapeutic strategies targeting AKR1C2 may need to be tailored based on the sex of the patient.

4.3. Clinical implications and Potential Therapeutic Strategies

The pharmacological interactions of AKR1C enzymes have garnered substantial interest in recent years, given their critical role in metabolic processes associated with prostaglandins, steroid hormones, and cytotoxic agents. Moreover, functioning as a phase I detoxifying enzyme, AKR1C possesses the capability to neutralize harmful components present in tobacco smoke, such as nicotine-derived nitrosamine ketones (NNKs). However, alcohol and tobacco consumption did not affect AKR1C2 expression or prognosis in OPSCC[108].

These enzymes facilitate reactions that directly influence cancer progression by activating oncogenic signaling pathways [89,90]. Despite the importance of inhibiting specific members of the AKR1C family, most of the available inhibitors act non-selectively, indiscriminately targeting all AKR1C isoforms. Key classes of such inhibitors include nonsteroidal anti-inflammatory drugs (NSAIDs), benzodiazepines, steroids, and flavonoids, which affect a broad range of metabolic processes [80-83].

Acetylsalicylic acid (aspirin), one of the most well-known NSAIDs, has demonstrated potent inhibitory effects on AKR1Cs. Its ability to reduce enzyme activity highlights the potential therapeutic benefits of NSAID-based treatments in cancers that exhibit AKR1C overexpression [79]. There are also more specific inhibitors available for the individual AKR1C isoforms. For example, AKR1C1 can be selectively inhibited by 3-bromo-5-phenylsalicylic acid and flufenamic acid [118-120], while AKR1C3 can be targeted by compounds such as tolfenamic acid and indomethacin [79]. Clinical trials, such as a Phase I/II trial investigating indoethacin in prostate cancer (NCT02935205) [121], are already investigating the use of these selective inhibitors for therapeutic applications.

Despite recent developments, a selective AKR1C2 inhibitor remains elusive; however, its expression can be diminished by ursodeoxycholic acid (USDC). This compound demonstrated promising cooperative effects when used in combination with cisplatin, the primary chemotherapeutic agent for OPSCC, in studies conducted on cell lines [120]. Combining these therapeutic approaches may enhance treatment efficacy, particularly for cancers managed with cisplatin-based medications such as cis-diamminedichloroplatinum(II) (CDDP). This is relevant for OPSCC tumor stages, where curative treatment includes radiochemotherapy, as well as for advanced metastatic or recurring tumor stages, where CDDP-based chemotherapy is considered a palliative care option.

The use of NSAIDs as a strategy to inhibit AKR1C activity is further supported by studies on HNSCC, which harbor mutations or amplifications in the PIK3CA gene. As mentioned previously, this gene is commonly associated with AKR1C overexpression and is linked to the activation of the PI3K/AKT signaling pathway, which promotes tumor growth and survival [89,90]. Research has shown that patients with these genetic alterations who regularly use NSAIDs for six months or longer experience a significant improvement in disease-specific survival [122,123]. This suggests that NSAIDs may be particularly beneficial in cancers characterized by dysregulated AKR1C expression.

Furthermore, findings from the Nurses' Health Cohort Study provide additional evidence for the efficacy of NSAIDs in cancer treatment. In this study, the use of both aspirin and non-aspirin NSAIDs was associated with prolonged survival in patients with ovarian cancer receiving cisplatin chemotherapy [124]. This further emphasizes the promise of using NSAIDs in combination with established cancer drugs to improve treatment efficacy, especially in malignancies in which AKR1C enzymes and associated metabolic processes are key contributors to CDDP resistance and cancer progression.

4.4. Conclusion and outlook

This study provides an in-depth exploration of aldo-keto Reductase 1C2 (AKR1C2) expression and regulation in both HPV-positive and HPV-negative oropharyngeal squamous cell carcinoma (OPSCC), offering critical insights into its role in cancer progression. The findings reveal a strong link between high AKR1C2 expression and positive HPV status, as well as advanced AJCC tumor classification, which suggests that AKR1C2 may actively contribute to tumor growth and aggressiveness. In HPV-positive OPSCC, the interplay between viral mechanisms and AKR1C2-associated pathways is particularly striking, potentially contributing to enhanced tumor formation.

A significant finding of this investigation is the discovery that AKR1C2 expression affects patient outcomes differently based on sex. While elevated AKR1C2 levels were associated with worse survival rates in male patients, the opposite effect was observed in female patients, where higher AKR1C2 expression was correlated with improved overall survival. This distinction emphasizes the need to consider sex-specific biological differences in prognosis and treatment strategies for OPSCC. Hormonal influences, immune responses, and lifestyle factors, such as smoking and alcohol consumption, which are known to affect hormone metabolism and cancer susceptibility, may partially explain these sex-specific differences. For instance, estrogen pathways in women may interact with AKR1C2 expression, potentially providing a protective effect that is not observed in male patients. This finding highlights the importance of tailoring treatment approaches according to sex and individual biological factors.
Another important discovery is the involvement of AKR1C2 in managing oxidative stress. AKR1C2, along with other enzymes in the AKR1C family, is known to detoxify harmful substances, such as nicotine-derived nitrosamine ketones (NNKs), which are present in tobacco smoke. This detoxifying function protects cells from the oxidative damage caused by external stressors. AKR1C2 has also been implicated in reducing the effectiveness of chemotherapeutic agents such as cisplatin, leading to treatment resistance in tumors. Although AKR1C2 plays a role in detoxification, its expression and impact on prognosis in OPSCC patients were not influenced by lifestyle factors such as alcohol consumption and smoking. This suggests that the regulation of AKR1C2 expression involves more intricate, cancer-specific mechanisms, rather than being solely determined by external environmental exposure. Further investigation of the genetic or epigenetic factors that may regulate AKR1C2 expression across various patient subgroups is essential for understanding its role in tumor behavior and treatment response. Identifying these underlying mechanisms could reveal specific regulatory pathways that contribute to AKR1C2 dysregulation, offering new insights into personalized treatment approaches.
The implications of these findings are profound, particularly in the context of personalized cancer therapies. AKR1C2 could serve as a potential biomarker for identifying patients at higher risk of aggressive tumor behavior, especially in HPV-positive cases. Targeted inhibition of AKR1C2 may lead to new pharmacological approaches because current inhibitors such as NSAIDs and flavonoids are not highly specific and affect multiple AKR1C family members. However, AKR1C2 inhibition, such as by USDC, has shown promise in increasing the efficacy of chemotherapeutics such as cisplatin in certain cell lines. These findings suggest that combining existing chemotherapies with AKR1C2 inhibitors could potentially reduce resistance in some cancers.

Despite these promising insights, this study had several limitations. Owing to the relatively small sample size, especially the low number of female participants, larger studies are needed to confirm these findings. Further research is needed to explore how AKR1C2 evaluation can be incorporated into routine diagnostic procedures and to investigate the potential of AKR1C2 inhibitors as therapeutic agents in clinical settings. Additionally, the broader role of AKR1C family members in cancer biology, including their interactions with oxidative stress and detoxification pathways, warrants further investigation to fully understand their contribution to cancer progression and resistance to therapy.

We are currently investigating how viral proteins E6, E6*I, and E7 influence oxidative stress responses in cell culture models utilizing HEK293cell line and HNSCC cell lines such as UM-SCC-104, UPI:SCC090 and UPI:SCC152. In this research, GFP fusion proteins are employed to enable colocalization studies with additional markers. Moreover, the cells are subjected to incubation in both physiological (5% O_2) and hypoxic (2% O_2) atmospheres, simulating the oxidative stress conditions characteristic of the tumor microenvironment. The GFP tag facilitates the tracking of viral protein interactions with cellular components. Additionally, we are testing the effects of compounds already available as AKR1C inhibitors, such as NSAIDs, in vitro, aiming to gain further insights into their roles in modulating the oxidative stress response.

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6. Appendix

6.1. List of Illustrations

8. Pre-publication of results

1. **Ziogas M**, Seraphim G, Balaji H, Siefer O, Klussmann JP, Huebbers C. Analyse der Zellproliferation unter Einfluss von HPV16-E6*I-Expression, oxidativem Stress, 17β-Estradiol und AKR1C-Hemmung bei HPV-positivem OPSCC. Jahrestagung der Vereinigung Westdeutscher Hals-Nasen-Ohren-Ärzte 2024. Köln: Vereinigung Westdeutscher HNO-Ärzte. Jahrestagung der Vereinigung Westdeutscher Hals-Nasen-Ohren-Ärzte, German Medical Science GMS Publishing House; 2024.
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