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Direktor: Universitätsprofessor Dr. med. J. P. Klußmann

# Comprehensive analysis of VEGFR2 expression in HPV-positive and -negative oropharyngeal squamous cell carcinomas

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Mein geleisteter Beitrag an der vorliegenden Arbeit umfasst die Durchführung der immunhistochemischen Inkubationen (Avidin-Biotin-Peroxidase Methode und doppelte Immunfluoreszenz Methode) mit den Markern VEGFR2 und AKR1C3 sowie CD31, $p16^{INK4A}$, p53 und ALDH1A1. Die angegebenen Methoden sind nach entsprechender Anleitung durch Herrn Dr. med. dent. Yüksel Korkmaz von mir selbst ausgeführt worden. Die licht- und konfokalmikroskopischen Aufnahmen der jeweiligen Präparate wurden durch mich erstellt. Die densitometrische Quantifizierung der immunhistochemischen Signale wurde von mir selbst durchgeführt und der Datensatz von mir selbst ausgewertet.

Die immunhistochemische Inkubation mit dem Marker NRF2 ist durch Herrn Dr. rer. nat. Oliver G. Siefer und die Quantifizierung der NRF2 und AKR1C3-Färbungen durch Herrn PD Dr. Christian U. Hübbers im Jean-Uhrmacher-Institut erfolgt.

Die dieser Arbeit zugrunde liegenden Methoden der Zellkultivierung, retroviralen Transduktion und RT-qPCR wurden ohne meine Mitarbeit in dem Institut für Virologie von Frau Dr. Hanife Güler Dönmez durchgeführt. Die RT-qPCR Daten wurden von Herrn PD Dr. Christian U. Hübbers im Jean-Uhrmacher-Institut ausgewertet.

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## Abbreviations

| | |
|---|---|
| ABC | avidin-biotin-peroxidase-complex |
| AKR1C3 | aldo-keto-reductase 1C3 |
| ALDH1A1 | aldehyde dehydrogenase family 1 member A1 |
| ARE | antioxidant response element-like |
| β-Trcp1 | beta-transducin repeat containing E3 ubiquitin protein ligase |
| CDK | cyclin-dependent kinase |
| CDKN2A | cyclin-dependent kinase inhibitor 2A |
| CD31 | platelet endothelial cell adhesion molecule-1, PECAM-1 |
| CSC | cancer stem cell |
| Cul3 | Cullin-3 |
| DAB | 3,3‘-diaminobenzidine |
| DU | densitometrical units |
| EDTA | ethylenediaminetetraacetic acid |
| EGFR | epidermal growth factor receptor |
| EMT | epithelial-mesenchymal transition |
| eNOS | endothelial nitric oxide synthase |
| EC | endothelial cell |
| ERK | extracellular-signal-regulated kinase |
| FFPE | formalin-fixed paraffin-embedded |
| FGF | fibroblast growth factor |
| GLUT1 | glucose transporter 1 |
| H&E | hematoxylin and eosin |
| HIF-1α | hypoxia-inducible factor-1 α |
| HNSCC | head and neck squamous cell carcinoma |
| HPV | human papillomavirus |
| HSPG | heparin sulphate proteoglycan |
| IgG | immunoglobulin G |
| IHC | immunohistochemistry |
| KDR | kinase insert domain receptor (VEGFR2) |
| Keap1 | Kelch-like ECH-associated protein 1 |
| MAPK | mitogen-activated protein kinase |
| mRNA | messenger RNA |
| mTOR | mechanistic Target of Rapamycin |
| NAD(P+) | nicotinamide adenine dinucleotide phosphate |
| NO | nitric oxide |
| NRF2 | nuclear factor erythroid 2-related factor 2 |

| | |
|---|---|
| OPSCC | oropharyngeal squamous cell carcinoma |
| OS | overall survival; oxidative stress |
| p53 | tumour protein p53 |
| PBS | phosphate buffer saline |
| PCR | polymerase chain reaction |
| PD-1 | programmed death protein 1 |
| PDGF | platelet derived growth factor |
| PI3K/AKT-signalling | phosphatidylinositol-3-kinase/protein kinase B-signalling |
| PLC$\gamma$ | phospholipase C$\gamma$ |
| PlGF | placental growth factor |
| pRB | retinoblastoma protein |
| RBX1 | Ring-Box 1 |
| ROI | region of interest |
| ROS | reactive oxygen species |
| RT-qPCR | quantitative reverse transcription-PCR |
| SD | standard deviation |
| Src | proto-oncogene tyrosine-protein kinase Src |
| STATs | signal transducer and activator of transcription proteins |
| TBS | tris-buffer (2-amino-2-(hydroxymethyl)propane-1,3-diol) saline |
| TC | tumour cell |
| TKI | tyrosine-kinase inhibitor |
| Tris-HCl | tris-hydrochloride |
| VEGF | vascular endothelial growth factor |
| VEGFR | vascular endothelial growth factor receptor |

# 1. Summary

A rising proportion of head and neck squamous cell carcinomas (HNSCC) localised in the oropharynx (OPSCC) is associated with human papillomavirus (HPV) infections with HPV16 being the most prevalent type. Based on their differing risk factors, clinicopathological presentation, biological profiles, mutation patterns, expression signatures and presence of HPV DNA, OPSCC are subdivided into HPV-positive and -negative entities. However, modified treatment regimens that take HPV status into account have not yet been implemented in routine clinical practice. Strategies to interact with the vascular supply are promising therapeutic approaches in cancer treatment and have been studied over the past decades. Among the vascular endothelial growth factor receptors (VEGFR), VEGFR2 plays a decisive role in tumour angiogenesis. However, VEGFR2 is not only expressed on endothelial cells but can also be observed in tumour cells. Though it is known that VEGFR2 is commonly overexpressed in HNSCC, the influence of HPV on VEGFR2 in OPSCC is still unknown.

The present study addressed the question if differences in VEGFR2 expression in HPV-positive and -negative OPSCC exist and aimed to investigate the impact of HPV status on the quantitative and qualitative expression of VEGFR2 in OPSCC. Therefore, a series of 56 OPSCC samples with known HPV status was analysed. VEGFR2 expression patterns both in blood vessels from tumour-free and tumour-containing regions and within tumour cells were determined by immunohistochemistry. VEGFR2 signal intensities were quantified by densitometry and compared between HPV-positive and -negative OPSCC. Differences in subcellular colocalisation of VEGFR2 with endothelial (CD31), tumour cell ($p16^{INK4A}$ or p53) and cancer stem cell markers (ALDH1A1) were determined by double-immunofluorescence imaging.

The VEGFR2 expression was significantly reduced in HPV-positive tumour cells compared to HPV-negative OPSCC. However, with respect to the vascular supply, upregulation in tumour-containing regions was observed only in HPV-positive OPSCC. Furthermore, a strong colocalization of CD31 with VEGFR2 was observed in capillaries of HPV-positive OPSCC. Moreover, colocalization signals of ALDH1A1 with VEGFR2 in cancer stem cells were frequently observed in HPV-positive OPSCC, but sparsely detected in HPV-negative OPSCC. These results may suggest different routes of VEGFR2 signalling depending on HPV status and possibly triggered by hypoxia. While in HPV-positive OPSCC VEGFR2 might be associated with increased angiogenesis, in HPV-negative tumours an autocrine loop might regulate tumour cell survival and invasion. It appears that VEGFR2 expression may play a regulatory role in cancer stem cells of HPV-positive OPSCC. Future studies should clarify the molecular basis of mechanisms involved in the different regulation of VEGFR2 expression in OPSCC.

## Zusammenfassung

Ein steigender Anteil von Plattenepithelkarzinomen des Kopfes und Halses (HNSCC), die im Oropharynx (OPSCC) lokalisiert sind, ist mit Infektionen mit humanen Papillomaviren (HPV) assoziiert, wobei HPV16 der am häufigsten vorkommende Typ ist. Basierend auf den unterschiedlichen Risikofaktoren, der klinisch-pathologischen Präsentation, den biologischen Profilen, den Mutationsmustern, den Expressionssignaturen und dem Vorhandensein von HPV-DNA werden die OPSCC in HPV-positive und -negative Entitäten eingeteilt. Geänderte Behandlungsschemata, die den HPV-Status berücksichtigen, sind jedoch in der klinischen Routinepraxis noch nicht eingeführt worden. Strategien zur Interaktion mit der Gefäßversorgung sind vielversprechende therapeutische Ansätze in der Krebsbehandlung und wurden in den letzten Jahrzehnten untersucht. Unter den vaskulären endothelialen Wachstumsfaktor-Rezeptoren (VEGFR) spielt VEGFR2 eine entscheidende Rolle bei der Tumorangiogenese. VEGFR2 wird jedoch nicht nur auf Endothelzellen exprimiert, sondern kann auch in Tumorzellen beobachtet werden. Obwohl bekannt ist, dass VEGFR2 in HNSCC häufig überexprimiert wird, ist der Einfluss von HPV auf VEGFR2 in OPSCC noch unbekannt. Die vorliegende Studie ging der Frage nach, ob es Unterschiede in der VEGFR2-Expression bei HPV-positiven und -negativen OPSCC gibt und hatte zum Ziel, den Einfluss des HPV-Status auf die quantitative und qualitative Expression von VEGFR2 bei OPSCC zu untersuchen. Daher wurde eine Serie von 56 OPSCC-Proben mit bekanntem HPV-Status analysiert. Die VEGFR2-Expressionsmuster sowohl in Blutgefäßen aus tumorfreien und tumorhaltigen Regionen als auch innerhalb von Tumorzellen wurden immunhistochemisch bestimmt. Die VEGFR2-Signalintensitäten wurden densitometrisch quantifiziert und zwischen HPV-positiven und -negativen OPSCC verglichen. Unterschiede in der subzellulären Kolokalisation von VEGFR2 mit Endothel- (CD31), Tumorzell- ($p16^{INK4A}$ oder p53) und Krebsstammzellmarkern (ALDH1A1) wurden mittels Doppel-Immunfluoreszenz-Bildgebung bestimmt.

Die VEGFR2-Expression war in HPV-positiven Tumorzellen im Vergleich zu HPV-negativen OPSCC signifikant reduziert. In Bezug auf die vaskuläre Versorgung wurde jedoch nur bei HPV-positiven OPSCC eine Hochregulierung in tumorhaltigen Regionen beobachtet. Weiterhin wurde eine starke Kolokalisation von CD31 mit VEGFR2 in Kapillaren von HPV-positiven OPSCC beobachtet. Darüber hinaus wurden bei HPV-positiven OPSCC häufig Kolokalisationssignale von ALDH1A1 mit VEGFR2 in Krebsstammzellen beobachtet, während sie bei HPV-negativen OPSCC nur spärlich vorhanden waren.

Diese Ergebnisse deuten darauf hin, dass die VEGFR2-Signalübertragung je nach HPV-Status unterschiedlich verläuft und möglicherweise durch Hypoxie ausgelöst wird. Während in HPV-positiven OPSCC VEGFR2 mit einer erhöhten Angiogenese assoziiert ist, könnte in HPV-negativen Tumoren eine autokrine Schleife das Überleben und die Invasion der

Tumorzellen regulieren. Es scheint, dass die VEGFR2-Expression eine regulatorische Rolle in Krebsstammzellen von HPV-positiven OPSCC spielen könnte. Zukünftige Studien sollten die molekulare Basis der Mechanismen klären, die an der unterschiedlichen Regulation der VEGFR2-Expression bei OPSCC beteiligt sind.

# 2. Introduction

Among head and neck tumours, oropharyngeal squamous cell carcinomas (OPSCC) show a steadily increasing annual incidence.[1-5] Up to 70% of OPSCC are associated with human papillomavirus (HPV) primarily of type 16.[6] HPV-positive and HPV-negative OPSCC show different mutation patterns and expression signatures.[7,8]

Regarding the hallmarks of cancer, angiogenesis is one characteristic which is essential for tumour growth and spread.[9,10] Angiogenesis is modulated and controlled upon others by vascular endothelial growth factor signalling.[11] The vascular endothelial growth factor receptor 2 (VEGFR2; also known as KDR, kinase insert domain receptor) is expressed in tumour cells and endothelial cells. It plays a crucial role in angiogenesis, as its activation initiates signalling pathways that regulate endothelial cell migration, proliferation and motility.[12] In head and neck tumours in general, tumour cells overexpress VEGFR2.[13] It is suggested that an autocrine loop promotes tumour cell proliferation, motility, invasion and survival.[14]

Research in tumours and cell lines from the cervix uteri have shown, that in the presence of HPV the expression of the growth factor VEGF can be modulated by the viral oncoproteins E6 and E7.[15,16] However, the effects of HPV on VEGFR2 expression in oropharyngeal cancer cells and their surrounding tissue, including blood vessels, remain unclear.

## 2.1 Oropharyngeal squamous cell carcinomas

Tumours of the head and neck belong to the top ten of the most common tumours and about a half-million new cases per year worldwide can be registered. Mostly these are squamous cell carcinomas (HNSCC).[17] Since the typical risk factors nicotine and alcohol consumption for developing head and neck cancer have denoted a decline, the incidence of HNSCC has also been decreasing, except a subgroup of HNSCC in the oropharynx associated with HPV infections.[1-5] Based on different clinical-pathological as well as biological profiles, including cellular and molecular tumour characteristics, the separation between HPV-positive and -negative OPSCC is validated.[1-4,18,19] Both are considered as two different tumour entities, which is reflected by the update of the 7th TNM classification into the 8th TNM classification by different classifications of N status.[20]

In the USA, HPV-positive OPSCC had already increased from 16.3% (1984-1989) to 70% in the years 2000-2004.[21] Recent data from the USA show that the relative incidence of HPV-associated OPSCC had already exceeded that of cervical cancers in women.[18] In Europe, this trend of increasing incidence is also foreseeable, but not yet as strong as in the USA, with higher prevalence in Northern Europe (57%) than in Southern Europe (24%),[4,18] In Germany, the HPV prevalence in OPSCC is around 40%.[4] It is assumed that the increasing prevalence of HPV-associated squamous cell carcinoma in the oropharynx is due to altered sexual

behaviour. The main risk factors for transmission include frequent partner change and oral sex.[5,22]

### 2.1.1. Carcinogenesis in HPV-positive and HPV-negative OPSCC

Today, more than 200 HPV types are identified. The distinction between high-risk and low-risk HPV types is particularly relevant. The low-risk types include types 1, 3, 4, 6 and 11. These are responsible for the development of condylomas and genital warts as well as for respiratory papillomatosis. The high-risk HPV types are associated with carcinoma formation and include the types 16, 18, 33, 35, 48 and 51. Frequently (85%) associated with OPSCC are types 16 and 18, with type 16 being the most prevalent.[4,23]

OPSCC occur basically within four subareas of the oropharynx: tonsils, base of tongue, palate and other oropharynx. Frequently affected by HPV infections are the tonsils, followed by the base of the tongue, and less so other areas of the oropharynx[4,5]. Specialized epithelia in particular, such as the reticular crypt epithelium of the tonsils, are preferentially infected by human papillomaviruses as they do not have a strong barrier function and have an increased occurrence of epithelial stem cells.[23,24] Human papillomaviruses infect the basal epithelial cells through microlesions or porous, thin skin layers.[23]

If the HPV DNA reaches the cell, the viral L1 capsid binds to exposed heparin sulphate proteoglycans (HSPGs) in serum at micro-injury sites during the wound healing process, and simultaneously the virion binds to $\alpha$6 integrins, leading to several signalling events. Conformational changes of the HSPG, L2 cleavage and binding of the exposed L2 N-terminus to an L2-specific receptor subsequently drive endocytosis of HPV16.[24] Then the HPV DNA enters the nucleus, persists as an extrachromosomal episome and may integrate into the host genome over time. The integration takes place primarily in promoter regions of important cell regulatory gene segments.[1,4] While viral integration is essential for the process of carcinogenesis in cervical cancer, for OPSCC, it has been shown that the expression level of HPV interrupted human gene transcripts is independent of the integration status. In addition, episomal or mixed gene transcripts are more common.[25] However, metabolic pathways are often deregulated in OPSCC with integrated viral DNA.[26]

As host cells differentiate and reach higher epithelial layers, the replication cycle progresses. HPV is almost inactive in the basal layer. In the upper layers, the viral oncoproteins E2, E6 and E7 are first to be active. Finally, in the uppermost layers, the late proteins L1 and L2 are responsible for virus replication.[23] The interaction of viral oncoproteins with regulatory proteins causes cellular alterations leading to tumour progression. Interactions of E6 with p53 lead to proteolytic degradation of p53 and consequently to the prevention of apoptosis. E7 deregulates the pRB cascade resulting in uncontrolled progression of the cell cycle and going along with overexpression of the regulatory protein $p16^{INK4A}$ (whose effect is abolished by inactivation of

pRB downstream). Therefore, $p16^{INK4A}$ is used for diagnostic purposes. It indicates HPV-associated carcinogenesis and is inactivated in HPV-negative OPSCC.[1,4]

Unlike HPV-positive OPSCC, HPV-negative OPSCC develop from premalignant precursor lesions. Risk factors for the development of these tumours are the classical carcinogens smoking in combination with alcohol consumption. Accumulation of somatic mutations, other genetic alterations like gene amplification or loss and epigenetic changes lead to activation of oncogenes and inactivation of tumour suppressor genes, resulting in invasive carcinoma development.[3] In HPV-negative OPSCC characteristic genetic alterations are mutations of the TP53 gene and loss of cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, encoding the p16INK4A protein, which disrupts cyclin D-CDK complexes of the cell cycle. In addition, frequently observed amplification of cyclin D1 (CCND1) contributes to unscheduled DNA replication.[2,3] In response, p53 protein would induce the CDK inhibitor p21 and apoptosis, but TP53 gene is often inactivated or a p53 dominant-negative mutant is overexpressed, leading to unrestrained proliferation of tumour cells and prevention from apoptosis.[1-3]

### 2.1.2. Prognosis and therapy

Due to the occurrence of frequent locoregional recurrences, distant metastases and second primary tumours, HPV-negative HNSCC show aggressive behaviour. Therefore, morbidity and mortality are high.[3,27] HPV-positive OPSCC patients, on the other hand, have fewer genetic alterations, lower alcohol and nicotine consumption, younger average age and thus fewer comorbidities, which is associated with a better prognosis for this type of OPSCC.[19,21,24,27] Despite the characteristics that HPV-positive OPSCC tend to present as poorly differentiated carcinomas and the frequent presentation with relatively small tumour size (low T-stage) but significant lymph node involvement (high N-stage), overall these factors result in HPV-positive OPSCC having a better prognosis (80% 5-year survival rate) than HPV-negative ones (40-50% 5-year survival rate).[1,5,27-29] In addition, they respond better to radiochemotherapy on account of an intact apoptotic response, and these therapies probably trigger an immune response against viral antigens.[4,5,24] Also, HPV-positive OPSCC patients display a lower risk of locoregional recurrences and second primary neoplasia than HPV-negative OPSCC patients after postoperative chemoradiation, which are risks more likely to be caused by field carcinogenesis.[3,17,27] However, HPV-associated carcinomas have a higher risk for metastasis, possibly driven by an increase in the number of cancer stem cells (CSC).[30]

The improved prognosis of HPV-associated OPSCC has been taken into account with the current TNM classification, which is intended to enable a de-escalation of the therapy.[20] However, modified treatment recommendations that take the HPV status of the tumour into account have been lacking in clinical practice.[2,27] Depending on localisation and size, the current treatment options for OPSCC include surgery, radiation, chemotherapy and targeted therapy.[4,20,27] OPSCC in early stages can be treated by surgical resection and/or

radiotherapy.[27] In locally advanced stages, the tumour is treated either with definitive chemoradiotherapy or surgically with adjuvant radiotherapy and, if necessary, additional chemotherapy in case of unfavourable pathological findings. The choice of procedure is based on the operability and the expected benefit of the patient.[4,27]

The first available targeted therapy was the antibody cetuximab against the epidermal growth factor receptor (EGFR) and is used in combination with radiotherapy in metastatic or recurrent HNSCC.[17,31,32] Nivolumab and pembrolizumab (both programmed death protein 1 (PD-1) inhibitors) are two further approved targeted therapeutics for relapsed or metastatic HNSCC with disease progression during or after platinum-containing chemotherapy.[33]

Nevertheless, the consequences of (chemo-)radiation are not insignificant for the quality of life, as patients often suffer from swallowing difficulties, xerostomia and taste disorders.[1,5] The aim should be, therefore, to make targeted use of these molecular differences between HPV-driven and non-HPV-driven OPSCC for therapy to improve oncological outcomes in terms of reducing treatment-related toxicities and functional impairment and improving quality of life without compromising survival.[27]

## 2.2 Tumour angiogenesis

Oxygen supply is crucial for tumour growth and must therefore be ensured by the formation of new supporting blood vessels. The mechanisms involved include vasculogenesis, angiogenesis and vascular mimicry.[34-36] Vasculogenesis is the formation of entirely new blood vessels from endothelial precursor cells. Angiogenesis instead, is blood vessel formation from pre-existing blood vessels and is subdivided into splitting and sprouting angiogenesis.[35] Sprouting angiogenesis is commonly found in outgrowing tumours. Tumour cells can arrange to form tubular structures, which mimic capillaries and is therefore called vascular mimicry.[36]

Tumour vasculature differs from normal vasculature in several ways. It is more immature, less organised, and the vessels have a fragile basement membrane, which leads to leaky and therefore, highly permeable vessels.[34,37] In order to guarantee a sufficient blood supply, growth factors stimulate tumour angiogenesis.[38]

### 2.2.1. Regulation of tumour angiogenesis

There are many growth factors involved in the process of angiogenesis such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs) and platelet-derived growth factors (PDGFs).[35] In summary, vascular endothelial growth factor (VEGF) is the most important among them involved in tumour angiogenesis.[39]

VEGF secreting sources in the tumour milieu are, beyond endothelial cells, particularly tumour cells and tumour associated macrophages. Upon VEGF binding, the major responses include endothelial cell proliferation and migration, resulting in tube formation, survival and vascular permeability.[35,37] The VEGF protein family consists of the glycoproteins VEGF-A (generally

regarded as VEGF), VEGF-B, VEGF-C and VEGF-D. Viral VEGF-E and placental growth factor (PlGF) also belong to the VEGF protein family. VEGFs are generally responsible for the formation, function and maintenance of vessels, while VEGF-A, in particular, contributes to the main (tumour) angiogenesis responses.[11,37] The various VEGF ligands bind to different types of VEGFRs because of distinct patterns of receptor specificity.[40]

VEGF receptors (VEGFRs) are tyrosine type III kinases. Three different VEGFR types are known: VEGFR1, VEGFR2, and VEGFR3. Besides endothelial cells, VEGFR1 is localised on various immune cells and is regarded as a decoy receptor.[41] It has reduced kinase activity, so it limits the amount of free available VEGF ligand.[42] VEGFR2 activation in blood vessels stimulates angiogenesis. A detailed description of the functions and role of VEGFR2 is given in the following paragraphs. *"VEGFR3 is explicitly expressed in the lymph endothelium and is therefore crucial for lymph vessel formation.[43]"* [44]

The typical structure of receptor tyrosine kinases consists of an extracellular domain build of seven immunoglobulin-like domains, followed by a transmembrane domain, a juxtamembrane domain and a tyrosine kinase domain, which is interrupted by a short kinase insert domain. The cytoplasmatic c-terminal tail is a sequence carrying several tyrosine residues.[45] The second and third immunoglobulins of the extracellular domain build the ligand-binding region in VEGF receptors 1 and 2. The intracellular domain has several tyrosine residues which serve as docking regions for other downstream molecules (see next paragraph). Only VEGFR2 additionally has a tyrosine residue (Try 951) in the kinase insert domain, whose phosphorylation and subsequent signalling pathway is induced primarily in tumour angiogenesis.[11,46] The VEGFRs can be activated classically by ligand binding or by mechanosensory activation.[11] Downstream VEGFR signalling starts after receptor dimerisation and autophosphorylation of the tyrosine residues.

The main effects in tumour angiogenesis are induced upon binding of VEGF to the type 2 receptor.[45] VEGFR2 signalling plays a decisive role in tumour angiogenesis because its activation initiates signalling pathways that regulate endothelial cell migration, proliferation and motility. Further, it leads to an increase in nitric oxide (NO) production and thus regulates vascular tone and permeability.[11,12] The intracellular signalling pathways upon VEGFR2 activation include the PLC$\gamma$-ERK1/2 pathway, the PI3K-AKT-mTOR pathway, Src tyrosine kinases, small GTPases and kinases such as MAPK and STATs.[11] Stimulation of endothelial nitric oxide synthase (eNOS) regulates vascular permeability.[37]

### 2.2.2. Effects of HPV on tumour angiogenesis

Angiogenesis is a little-explored field in HNSCC and shows differences comparing HPV-positive and HPV-negative HNSCC. In immunohistochemical studies, the epidermal growth factor receptor (EGFR; associated with angiogenesis as a proximal factor due to its capability to induce VEGF transcription) shows higher expression levels in HPV-negative

OPSCC, whereas studies on VEGF expression in HNSCC according to HPV status present, however, with contrary associations.[47] Immunohistochemical studies investigating possible influences of HPV on the expression of VEGFR2 have not yet been conducted. Still, little is known about heterogeneity in angiogenesis of HPV-positive and HPV-negative OPSCC.

*"In vitro studies with HPV-positive and -negative cervical cancer cell lines have shown that the viral oncoproteins E6 and E7 induce VEGF-expression.[15,16] Although E6 degrades the transcription factor TP53, which acts as an angiogenic suppressor, E6 is capable of inducing VEGF in a TP53 independent manner by direct interaction with its promoter region.[15,32] Furthermore, both E6 and E7 promote Hypoxia inducible factor 1 alpha (HIF-1$\alpha$) expression, which can upregulate VEGF, while knockdown of HIF-1$\alpha$ has been shown to suppress angiogenic activity in viral oncogene expressing cells in vitro.[16,48]"* [44] It is suggested that the HPV oncoproteins E6 and E7 can also indirectly affect endothelial cell behaviour by causing alterations in the expression profile of angiogenic factors in keratinocytes. These HPV induced phenotype changes stimulate endothelial cell proliferation and migration.[49,50]

## 2.3 Role of VEGF/VEGFR2 signalling and oxidative stress in tumour cells

Many solid tumour types such as breast cancer, pancreatic cancer and HNSCC consist of VEGF overexpressing tumour cells. This overexpression might indicate, that the VEGF signalling is not only crucial for tumour vascularisation, but also for survival and proliferation of tumour cells themselves.[14] Consequently, expression of VEGFR2 has been shown for several tumour types, including HNSCC.[13] It is assumed, that an autocrine signalling loop exists, which, despite the proangiogenic effects, promotes tumour cell proliferation, motility and invasion.[51] Furthermore, the antiapoptotic effects of VEGF/VEGFR2 signalling contribute to tumour cell survival.[14] VEGF-overexpression is associated with increased tumour progression and poor prognosis due to increased resistance to chemotherapy. In addition, VEGF signalling can promote tumour initiation and oncogenic transformation. It favours the formation of cancer stem cells (CSC) and their self-renewal.[52]

*"Furthermore, VEGF expression is induced by the transcription factor nuclear factor erythroid-derived 2-like 2 (NFE2L2 / NRF2).[53] Upon oxidative stress (OS) stimuli, NRF2 dissociates from its cytosolic inhibitor complex consisting of Kelch-like ECH-associated protein 1(Keap1), Cullin-3 (Cul3) and Ring-Box 1 (RBX1) to translocate into the nucleus, where NRF2 binds to antioxidant response element-like (ARE) sequences in promoter regions of several target genes including VEGF. The role of NRF2 is to protect normal cells from damage induced by reactive oxygen species (ROS) and to contribute to tissue regeneration.[54] However, hyperactivation of NRF2 followed by overexpression of several of its target genes leads to exuberant protection against OS in malignant cells preventing apoptosis and cell death, eventually leading to resistance against radio- and chemotherapy.[26,53] In HPV-negative HNSCC, NRF2 overexpression is typically a consequence of mutations and copy number*

*variations in its own gene and in the genes Keap1, Cul3 and RBX1 encoding for its regulatory complex, whereas NRF2 deregulation is caused by viral proteins in HPV-positive tumours.[26,55,56] Aldo-Keto-Reductase 1C3 (AKR1C3) is one of these target genes upregulated by NRF2 that, in addition to its role in lipid metabolism, is a phase I detoxifying enzyme for numerous drugs, including platinum-type chemotherapeutic agents.[54] AKR1C3 expression is a useful read-out for detecting increased OS levels in various cancers including OPSCC, whereby overexpression correlates with significantly unfavourable survival.[26,54]"* [44]

### 2.3.1. Implications of autocrine VEGF/VEGFR2 signalling in CSCs

CSCs play a decisive role in the initiation of tumours, the recurrence of tumours and the development of metastases.[57] Defining features include the ability to self-renew, to differentiate into tumour progenitor cells and mechanisms that make them immortal like increased DNA repair capacity and high expression of multi-drug-resistance proteins. They can migrate to other tissues and initiate metastases, but account only for a small amount of the total tumour cells.[57,58]

To protect themselves from toxic elevated ROS levels, the CSCs overexpress ALDH1A1, which is an enzyme that functions as a detoxifying agent by reducing toxic aldehydes resulting from lipid peroxidation in cells undergoing oxidative stress.[59,60] ALDH1A1 belongs to NAD(P+)-dependent enzymes, which catalyse the oxidation of endogenous and exogenous aldehyde substrates to carboxylic acids. Besides, they can oxidise retinal and aliphatic aldehydes.[61] The ALDH1A1 enzyme activity provides low ROS and reactive aldehydes, which prevents CSC apoptosis.[62] Cancer cell-acquired drug resistance is associated with increased ALDH1 expression promoting tumour growth and initiating carcinogenesis in CSCs. Besides, ALDH1A1 is used as a CSC marker to determine CSCs and characterise enzymatic counteractivity against the formation of ROS in these cells.[61]

CSCs can be located in a perivascular niche and VEGF-A secreted by these cells functions in a paracrine way to stimulate angiogenesis. Autocrine VEGF signalling mediated by VEGFR2 promotes dedifferentiation and an epithelial-mesenchymal transition (EMT) phenotype.[52] EMT is the dynamic process of interconversion of epithelial cells to a mesenchymal cell state. The ability to switch between epithelial and mesenchymal phenotype by undergoing EMT results in phenotypic heterogeneity and plasticity of CSCs.[30] Cell changes involve disassembly of epithelial cell-cell junctions, dissolution of apical-basal cell polarity, increased motility and invasive capacities among others, so that depending on EMT activation malignant progression of carcinoma proceeds.[63]

## 2.4 Questions and aim of the work

VEGF signalling regulated by VEGFR2 plays a decisive role in tumour angiogenesis, initiation and progression in several tumours including HNSCC. However, nothing is known about the

expression pattern of VEGFR2 in OPSCC and whether there is an HPV-dependent effect on VEGFR2 expression. It is the first study to determine whether VEGFR2 intensity differs between HPV-positive and HPV-negative OPSCC, which may play a relevant role in the development of specific therapies.

In detail, the present study addressed the following questions:

1) Are there differences in the localisation and expression of VEGFR2 in blood vessels of HPV-positive and HPV-negative OPSCC?
2) Are there differences in the localisation and expression of VEGFR2 in tumour cells of HPV-positive and HPV-negative OPSCC?
3) Are there differences in the subcellular localisation of VEGFR2 in tumour cells and surrounding blood vessels of HPV-positive and HPV-negative OPSCC?
4) Is VEGFR2 localised in cancer stem cells of HPV-positive and HPV-negative OPSCC? Are there differences in expression of VEGFR2 in cancer stem cells of HPV-positive and HPV-negative OPSCC?
5) Is there a correlation of VEGFR2 expression concerning other clinical parameters?

The aim of this study was to evaluate whether HPV exert specific effects on VEGFR2 expression in OPSCC and thus possibly on the regulation of vascularisation.

## 3. Published original article

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*Article*

# Comprehensive Analysis of VEGFR2 Expression in HPV-Positive and -Negative OPSCC Reveals Differing VEGFR2 Expression Patterns

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**Simple Summary:** Up to 50% of oropharyngeal squamous cell carcinomas (OPSCC) are associated with human papillomavirus type 16 (HPV16), the annual incidence of which is steadily increasing. HPV-positive and -negative OPSCC exhibit a different biology, which is characterized by distinct mutation signatures and expression patterns. It is known that VEGFR2 is commonly overexpressed in HNSCC, but the influence of HPV on VEGFR2 in OPSCC is still unknown, although VEGFR2 has emerged as a promising target in tumor therapy. The aim of our study was to evaluate whether HPV exerts specific effects on VEGFR2 expression in OPSCC and thus possibly on the regulation of vascularization. Interestingly, while HPV-negative carcinoma upregulates VEGFR2 in tumor cells, in HPV-positive carcinoma VEGFR2 is upregulated in tumor-supporting blood vessels. HPV-positive OPSCC with high VEGFR2 expression is associated with poor prognosis, supporting the prognostic significance of deregulated VEGF signaling for OPSCC patients.

**Abstract:** VEGF signaling regulated by the vascular endothelial growth factor receptor 2 (VEGFR2) plays a decisive role in tumor angiogenesis, initiation and progression in several tumors including HNSCC. However, the impact of HPV-status on the expression of VEGFR2 in OPSCC has not yet been investigated, although HPV oncoproteins E6 and E7 induce VEGF-expression. In a series of 56 OPSCC with known HPV-status, VEGFR2 expression patterns were analyzed both in blood vessels from tumor-free and tumor-containing regions and within tumor cells by immunohistochemistry using densitometry. Differences in subcellular colocalization of VEGFR2 with endothelial, tumor and stem cell markers were determined by double-immunofluorescence imaging. Immunohistochemical results were correlated with clinicopathological data. HPV-infection induces significant downregulation of VEGFR2 in cancer cells compared to HPV-negative tumor cells ($p = 0.012$). However, with respect to blood vessel supply, the intensity of VEGFR2 staining differed only in HPV-positive OPSCC and was upregulated in the blood vessels of tumor-containing regions ($p < 0.0001$). These results may suggest different routes of VEGFR2 signaling depending on the HPV-status of the OPSCC. While in

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HPV-positive OPSCC, VEGFR2 might be associated with increased angiogenesis, in HPV-negative tumors, an autocrine loop might regulate tumor cell survival and invasion.

**Keywords:** vascular endothelial growth factor receptor 2; oropharyngeal squamous cell carcinoma; human papillomavirus; cancer stem cell

## 1. Introduction

A rising proportion of head and neck squamous cell carcinomas (HNSCC) localized in the oropharynx (oropharyngeal squamous cell carcinoma, OPSCC) is associated with human papillomavirus (HPV) infections with HPV16 being the most prevalent type [1]. Data from the United States allowed for the projection that OPSCC case numbers would overtake the number of cervical carcinomas in 2020 and data from the Centers for Disease Control (CDC) show that this was already the case in 2019 [1,2].

Based on their differing risk factors, clinicopathological presentation, biological profiles, mutation patterns and expression signatures, HPV-positive and HPV-negative OPSCC can be regarded as two distinct entities [3]. Accordingly, the recent TNM-Classification of OPSCC has been adapted and now distinguishes between $p16^{INK4A}$-positive (HPV-driven) and $p16^{INK4A}$-negative (HPV-negative) OPSCC, thus taking different treatment prognoses into account [4].

The vascular endothelial growth factor receptors (VEGFRs) are expressed on the cell surface and bind to the signaling ligand vascular endothelial growth factor (VEGF). VEGFR2 on endothelial cells is primarily involved in angiogenesis and plays a crucial role in tumor angiogenesis [5]. However, VEGFR2 is not only expressed on endothelial cells but can also be observed in tumor cells [6]. Apart from VEGFR2, two other VEGF receptors are described to be of clinical significance. VEGFR1 is localized on immune and endothelial cells and is considered as a decoy receptor that limits the amount of free available VEGF ligand. VEGFR3 is explicitly expressed in the lymph endothelium and is therefore crucial for lymph vessel formation [7]. Following binding of the VEGF ligand, the VEGF/VEGFR2 signal cascade is induced and leads to activation of pathways such as PLC-ERK1/2, PI3K-AKT-mTOR, of Src tyrosine kinases, small GTPases and kinases such as MAPK and STATs [5]. The activated signaling pathways regulate endothelial cell migration, cell proliferation and cell motility, and lead to an increase in nitric oxide (NO) production, thus regulating vascular tone and permeability [8]. VEGFR2-signalling is responsible for the formation, function and maintenance of vessels, all physiological processes which decisively contribute to the nutrient supply in healthy tissue as well as tumors [5]. Therefore, VEGFR2 is an important control node for tumor growth. Strategies to interact with the vascular supply in a therapeutic approach have been studied over the past decades, and VEGFR2 has emerged as a promising target in tumor therapy [8].

HNSCC, in general, present with overexpression of VEGFR2 and have the potential to create an autocrine loop, which is characterized by the tumor cells' ability to control their proliferation, motility, invasive capacity and survival in response to VEGFR2 expression [9,10]. However, the impact of HPV status on the quantitative and qualitative expression of VEGFR2 has not yet been investigated, although HPV may have specific effects on VEGFR2 expression. In vitro studies with HPV-positive and -negative cervical cancer cell lines have shown that the viral oncoproteins E6 and E7 induce VEGF-expression [11,12]. Although E6 degrades the transcription factor TP53, which acts as an angiogenic suppressor, E6 is capable of inducing VEGF in a TP53 independent manner by direct interaction with its promoter region [11]. Furthermore, both E6 and E7 promote Hypoxia-inducible factor 1 alpha (HIF-1$\alpha$) expression, which can upregulate VEGF, while knockdown of HIF-1$\alpha$ has been shown to suppress angiogenic activity in viral oncogene expressing cells in vitro [12].

Furthermore, VEGF expression is induced by the transcription factor nuclear factor erythroid-derived 2-like 2 (NFE2L2 / NRF2) [13]. Upon oxidative stress (OS) stimuli, NRF2 dissociates from its cytosolic inhibitor complex consisting of Kelch-like ECH-associated protein 1(Keap1), Cullin-3 (Cul3) and Ring-Box 1 (RBX1) to translocate into the nucleus, where NRF2 binds to antioxidant response element-like (ARE) sequences in promoter regions of several target genes including VEGF. The role of NRF2 is to protect normal cells from damage induced by reactive oxygen species (ROS) and to contribute to tissue regeneration [14]. However, hyperactivation of NRF2 followed by overexpression of several of its target genes leads to exuberant protection against OS in malignant cells preventing apoptosis and cell death, eventually leading to resistance against radio- and chemotherapy [13,15]. In HPV-negative HNSCC, NRF2 overexpression is typically a consequence of mutations and copy number variations in its own gene and in the genes Keap1, Cul3 and RBX1 encoding for its regulatory complex, whereas NRF2 deregulation is caused by viral proteins in HPV-positive tumors [15–17]. Aldo-Keto-Reductase 1C3 (AKR1C3) is one of these target genes upregulated by NRF2 that, in addition to its role in lipid metabolism, is a phase I detoxifying enzyme for numerous drugs, including platinum-type chemotherapeutic agents [14]. AKR1C3 expression is a useful read-out for detecting increased OS levels in various cancers including OPSCC, whereby overexpression correlates with significantly unfavorable survival [14,15].

## 2. Materials and Methods

### 2.1. Patient Material and Ethics Statement

A total of 56 OPSCC formalin-fixed paraffin-embedded (FFPE) tissue samples having both tumor-containing and adjacent tumor-free regions were available from the Departments of Otorhinolaryngology, Head and Neck Surgery, and Pathology, University of Cologne and were included in this study (Figure 1). Patients were treated between 2011–2013 at the Department of Otorhinolaryngology and Head and Neck Surgery, University of Cologne (Table 1). Primary tonsillar keratinocytes used in cell culture experiments were derived from routine tonsillectomy. The human ethics committee of the University of Cologne approved the procurement of human tumor tissue at surgery and performing research on this material (study number 11–346 for tumor tissue and 18–285 for keratinocytes). Patient material was handled according to the code for proper secondary use of human tissue, and written consent was obtained from all patients.

### 2.2. Tissue Fixation, Embedding and Sectioning

The tissue samples were collected in a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4, washed in 0.1 M PBS pH 7.4 at 4 °C and embedded in paraffin. These were then cut with a microtome to obtain 4-µm-thick sections.

### 2.3. DNA Isolation and HPV Typing

The DNA isolation and HPV typing procedures were performed by routine protocols as described previously including GP5+/GP6+ polymerase chain reaction (PCR) followed by direct sequencing and immunohistochemical staining against $p16^{INK4A}$ (Cintec, Roche, Freiburg, Germany), used as a surrogate marker for E7 expression [18].

### 2.4. Immunohistochemistry

Immunohistochemical stainings against the oxidative stress markers NRF2 and AKR1C3 were performed as described previously [15]. In brief, 4 µm-thick sections were deparaffinized and left in 0.01 M citrate buffer (pH 6.0) overnight at 70 °C for antigen retrieval. For detection of NRF2, polyclonal anti-NRF2 antibodies (HPA003097, Sigma-Aldrich, Taufkirchen, Germany; 1:200) together with its corresponding biotinylated secondary antibodies (Abcam, Cambridge, UK; 1:250) were used. AKR1C3 was detected by using monoclonal anti-AKR1C3 antibodies (A6229, Sigma, clone NP6.66.A6, 1:500) and corresponding biotinylated horse anti-mouse antibodies (Vector, Burlingame, CA, USA; 1:250).

Antigen retrieval of tissue samples for VEGFR2 staining was carried out by heating at 95 °C in 1mM EDTA buffer (pH 8.0) for 15 min. Slices were incubated with 0.3% $H_2O_2$ in 0.05 M Tris-buffered saline (TBS) for 20 min to inhibit endogenous peroxidase activity. This was followed by treatment with 0.25% Triton-X 100 detergent solution to block non-specific hydrophobic and non-specific ionic interactions. Non-specific immunoglobulin binding sites were blocked using a blocking solution containing 5% normal goat serum (Vector) and 2% bovine serum albumin (Sigma-Aldrich). For the detection of VEGFR2, sections were incubated overnight at 4 °C with rabbit anti-human monoclonal VEGFR2 antibodies (#2479, Cell Signaling Technology, Frankfurt a. M., Germany; clone 55B11, 1:500 in TBS). After incubation for 60 min with biotinylated goat anti-rabbit IgG (Vector, 1:300 in 0.05 M TBS) for antibody detection, subsequently, slides were incubated with avidin-biotin-peroxidase complex (ABC; Vectastain ABC kit, Vector) for 60 min. The peroxidase activity was developed for exactly 15 min with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) in 0.05 M Tris-HCl buffer (pH 7.6), containing 0.01% $H_2O_2$ and 0.01% nickel sulfate. Sections were mounted in xylene-based mounting medium Entellan (Merck, Darmstadt, Germany).

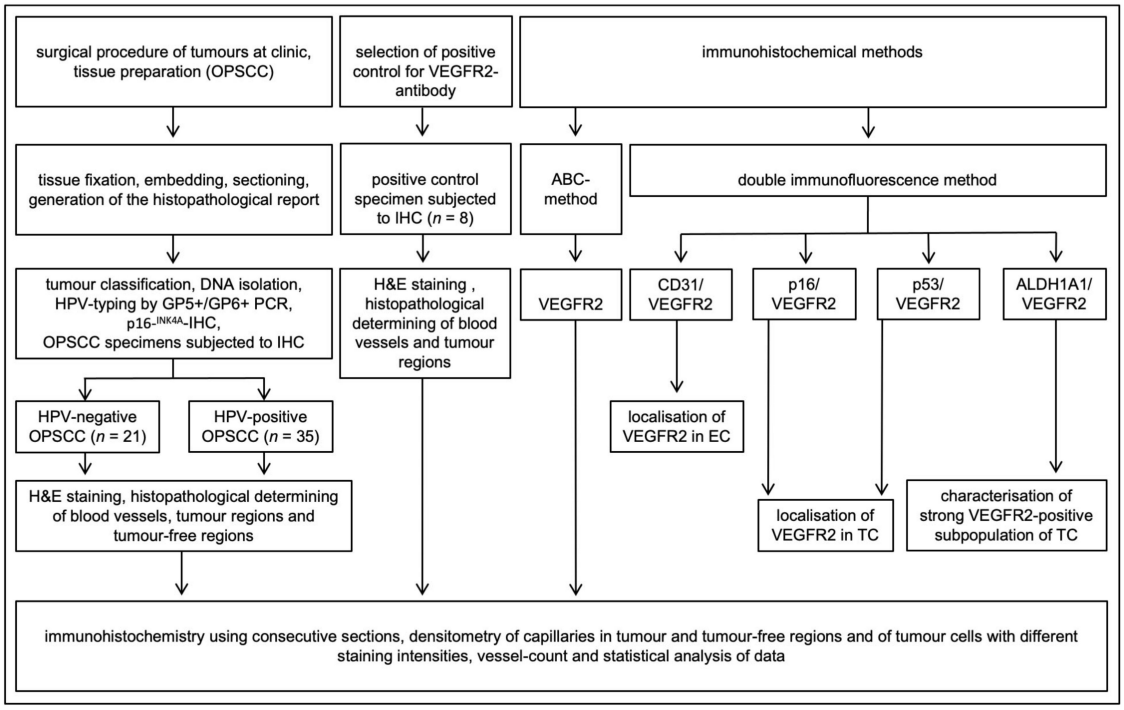


**Figure 1.** Schematic flow diagram of the study design and method. After surgery and tissue processing of OPSCC samples, the HPV-status was determined by GP5+/GP6+ polymerase chain reaction (PCR) and p16INK4A-immunohistochemistry (IHC). To test the specificity of the VEGFR2-antibodies, liver, melanoma, papillary thyroid carcinoma, and cervix squamous cell carcinoma tissues were selected as positive controls. First, hematoxylin and eosin (H&E) staining was performed to determine the regions relevant for further analyses. This was followed by staining of consecutive sections with VEGFR2-AB using the avidin-biotin-peroxidase complex (ABC). Subsequently, densitometric analyses of the VEGFR2-staining intensity of blood vessels in tumor regions and tumor-free regions, as well as tumor cells, were performed. The number of VEGFR2-expressing capillaries in a defined microscopic field was counted and statistically analyzed. Consecutive sections of OPSCC were selected for double immunofluorescence analysis to show colocalization of VEGFR2 and CD31 in endothelial cells (EC), to show the localization of VEGFR2 in tumor cells (TC) by double staining for p16INK4A- (in HPV-positive tumors) or for p53 (in HPV-negative tumors). Confocal double immunofluorescence analysis was performed for VEGFR2 and the stem cell marker ALDH1A1.

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

| Clinico Pathological Feature | Total | | HPV—Status | | | | | VEGFR2—Staining | | | | | NRF2—Staining | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | HPV—positive | | HPV—negative | | | VEGFR+ | | VEGFR2− | | | NRF2+ | | NRF2− | | |
| | n | % | n | % | n | % | $X^2$ | n | % | n | % | $X^2$ | n | % | n | % | $X^2$ |
| Mean age (years) | 60.2 | – | 59.9 | – | 60.5 | – | 0.838 | 59.2 | – | 61.0 | – | 0.517 | 62.9 | – | 58.8 | – | 0.177 |
| Gender | | | | | | | | | | | | | | | | | |
| Male | 43 | 76.8 | 26 | 46.8 | 17 | 30.0 | | 23 | 41.1 | 19 | 33.9 | | 17 | 30.3 | 26 | 46.4 | |
| Female | 13 | 23.2 | 9 | 16.1 | 4 | 7.1 | 0.747 | 2 | 3.6 | 11 | 19.6 | **0.023** | 2 | 3.6 | 11 | 19.6 | 0.098 |
| T classification | | | | | | | | | | | | | | | | | |
| pT1 and pT2 | 30 | 53.6 | 20 | 35.7 | 10 | 17.9 | | 13 | 23.6 | 16 | 29.1 | | 8 | 14.3 | 22 | 39.3 | |
| pT3 and pT4 | 26 | 46.4 | 15 | 26.8 | 11 | 19.6 | 0.584 | 12 | 21.8 | 14 | 25.5 | 1.00 | 11 | 19.6 | 15 | 26.8 | 0.265 |
| N classification | | | | | | | | | | | | | | | | | |
| pN0 | 14 | 25.0 | 9 | 16.1 | 5 | 8.9 | | 4 | 7.3 | 9 | 16.4 | | 4 | 7.1 | 10 | 17.9 | |
| pN1-3 | 42 | 75.0 | 26 | 46.4 | 16 | 28.6 | 1.00 | 21 | 38.2 | 21 | 38.2 | 0.341 | 15 | 26.8 | 27 | 48.2 | 0.751 |
| M classification | | | | | | | | | | | | | | | | | |
| pM0 | 54 | 98.2 | 35 | 63.6 | 19 | 34.5 | | 24 | 44.4 | 29 | 53.7 | | 18 | 32.7 | 36 | 65.5 | |
| pM1 | 1 | 1.8 | 0 | 0.0 | 1 | 1.8 | 0.364 | 0 | 0.0 | 1 | 1.9 | 1.00 | 1 | 1.8 | 0 | 0.0 | 0.345 |
| Death | | | | | | | | | | | | | | | | | |
| Yes | 16 | 28.6 | 9 | 16.1 | 7 | 12.5 | | 6 | 10.9 | 10 | 18.2 | | 12 | 21.4 | 4 | 7.1 | |
| No | 40 | 71.4 | 26 | 46.4 | 14 | 25.0 | 0.557 | 24 | 43.6 | 15 | 27.3 | 0.140 | 7 | 12.5 | 33 | 58.9 | **<0.0001** |
| HPV-status | | | | | | | | | | | | | | | | | |
| Negative | 21 | 37.5 | | | | | | 13 | 23.6 | 7 | 12.7 | | 7 | 12.5 | 14 | 25.0 | |
| Positive | 35 | 62.5 | | | | | | 12 | 21.8 | 23 | 41.8 | **0.048** | 12 | 21.4 | 23 | 41.1 | 1.00 |
| NRF2-staining | | | | | | | | | | | | | | | | | |
| Negative | 36 | 65.5 | 23 | 41.1 | 14 | 25.0 | | 14 | 25.5 | 22 | 40.0 | | | | | | |
| Positive | 19 | 34.5 | 12 | 21.4 | 7 | 12.5 | 1.00 | 11 | 20.0 | 8 | 14.5 | 0.256 | | | | | |
| AKR1C3-staining | | | | | | | | | | | | | | | | | |
| Yes | 38 | 67.9 | 25 | 44.6 | 13 | 23.2 | | 15 | 27.3 | 22 | 40.0 | | 14 | 25.5 | 22 | 40.0 | |
| No | 18 | 32.1 | 10 | 17.9 | 8 | 14.3 | 0.558 | 10 | 18.2 | 8 | 14.5 | 0.389 | 11 | 20.0 | 8 | 14.5 | 0.256 |
| Blood vessel density | | | | | | | | | | | | | | | | | |
| Low | 30 | 54.5 | 15 | 27.3 | 15 | 27.3 | | 12 | 21.8 | 18 | 32.7 | | 7 | 12.7 | 23 | 41.8 | |
| High | 25 | 45.5 | 20 | 36.4 | 5 | 9.1 | **0.027** | 13 | 23.6 | 12 | 21.8 | 0.424 | 12 | 21.8 | 13 | 23.6 | 0.087 |
| Smoking | | | | | | | | | | | | | | | | | |
| Never smoked | 13 | 23.2 | 12 | 21.4 | 1 | 1.8 | | 3 | 5.5 | 10 | 18.2 | | 3 | 5.4 | 10 | 17.9 | |
| Smoker | 43 | 76.8 | 23 | 41.1 | 20 | 35.7 | **0.020** | 22 | 40.0 | 20 | 36.4 | 0.110 | 16 | 28.6 | 27 | 48.2 | 0.507 |
| Alcohol | | | | | | | | | | | | | | | | | |
| No or < 1 glass/day | 43 | 78.2 | 30 | 53.6 | 14 | 25.0 | | 17 | 30.9 | 26 | 47.3 | | 17 | 30.4 | 27 | 48.2 | |
| Active, > 1 glass/day | 12 | 21.8 | 5 | 8.9 | 7 | 12.5 | 0.108 | 8 | 14.5 | 4 | 7.3 | 0.114 | 2 | 3.6 | 10 | 17.9 | 0.189 |

*n* = Number of patients. $X^2$: Chi-Square test for significance. For mean age, Anova is used to measure significance. Significant values are highlighted in bold.

Papillary thyroid carcinoma, liver tissue, melanoma and cervix carcinoma tissues ($n$ = 8) served as positive controls (Figure S1). Negative controls were performed without using primary antibodies to test the antibody specificities of the immunohistochemical reagents (Figures S2 and S3).

### 2.5. Double Immunofluorescence Labelling with VEGFR2, CD31, ALDH1A1, $p16^{INK4A}$ and TP53

Double immunofluorescence staining was performed separately in a subseries of consecutive sections using routine protocols published previously [19]. To validate that VEGFR2-positive cells located within blood vessels of HPV-positive and-negative OPSCC are endothelial cells, the samples were incubated with monoclonal anti-human CD31 (a gift from Prof. Dr. M. Koch, Cologne, 1:800) and monoclonal anti-VEGFR2 (1:500; 55B11 Cell Signaling Technology) antibodies. To prove that the VEGFR2 positive epithelial cells are tumor cells and to evaluate differences in the subcellular localization of VEGFR2 in tumor cells of OPSCC, HPV-positive samples were incubated with mouse anti-human polyclonal $p16^{INK4A}$ (1:50; BD Biosciences, Heidelberg, Germany) and VEGFR2 antibodies. HPV-negative samples were incubated with mouse anti-human polyclonal TP53 (Biologo, Kronshagen, Germany, 1:25) and VEGFR2 antibodies. To answer the question of whether individual cells, that are particularly immunoreactive to VEGFR2, represent a subpopulation of cancer stem cells, double staining of VEGFR2 with mouse anti-human monoclonal ALDH1A1 (1:500; H-4 (sc-374076) or B-5 (sc-374149) Santa Cruz, Heidelberg, Germany) was performed in a sub-series of sections (Supplementary Information 1). Subcellular localization patterns were analyzed by confocal microscopy (LSM 710, Carl Zeiss, Oberkochen, Germany).

### 2.6. Densitometric Quantification of Immunohistochemical Signals

The first slice of each sample was stained with hematoxylin and eosin to identify the tumor regions and blood vessels. The following slice of each sample was immunohistochemically incubated with VEGFR2 antibodies. Slides were digitalized in a slide scanner (Leica SCN 400) at 20x magnification. All analyses were carried out in a blinded manner with the bioimage software QuPath (version 0.1.3) [20]. Before the staining intensity was analyzed, the background staining was determined by setting color deconvolution values from a cell-free region of interest for every slide image, so that the program took the background grey values into account when measuring the staining intensities of endothelial or tumor cells. Prior to staining intensity analysis, the regions of interest (ROI) were determined and marked with the software tools. Due to a heterogeneous expression pattern of VEGFR2 within each sample, three different zones of three different staining intensity levels (low, moderate, high) were measured and the respective mean values were used for densitometry in tumor cells. For densitometry in blood vessels, three different vessels from the tumor region and three from the adjacent tumor-free region were selected and the mean intensity values were calculated for both. For the quantification of the blood vessel density, a microscopic field within the tumor region was determined by setting a grid size of 500 mm $\times$ 500 mm and the number of VEGFR2-positive vessels was counted within this field.

The quantification of immunohistochemical NRF2- and AKR1C3-stainings was performed as described previously [15]. In brief, tumor cells with positive nuclear staining against NRF2 were considered positive and a lack of staining was negative (Figure S4A,B). For the evaluation of the AKR1C3-staining, an index of staining intensities of the tumor tissue and the adjacent normal squamous epithelium was calculated. Tumors with higher expression intensity in the tumor compared to the surrounding normal tissue were evaluated as positive, those with the same or lower expression intensity in the tumor were evaluated as negative (Figure S4C,D).

### 2.7. Cell Culture and Retroviral Transduction

Primary human tonsillar keratinocytes isolated from routine tonsillectomy were cultivated in RM+medium (consisting of a 3:1 ratio of Dulbecco's modified Eagle's medium [DMEM]-F12 with 10% fetal calf serum [FCS], 1% glutamine, 0.4 µg hydrocortisone, $10^{-10}$ M cholera toxin, 5 µg/mL transferrin, $2 \times 10^{-11}$ M liothyronine, 5 µg/mL insulin, 10 ng/mL epidermal growth factor, 1 × penicillin–streptomycin mixture) [21]. Primary human foreskin keratinocytes were purchased from Lonza (Cologne, Germany, Cat.No. 00192907, Lot.No. 188311) and cultured in Keratinocyte Growth Medium 2 (PromoCell, Heidelberg, Germany). The OPSCC cell line FaDu and the retrovirus packaging cell line PT67 were maintained in DMEM with a 10% FCS and penicillin-streptomycin mixture. All cell lines were cultivated at 37 °C and 6% $CO_2$.

Transduction of cells with HPV16-E6, -E7 or -E6E7 coding retroviruses was performed as described previously [21–23]. The selection of infected cells was started 2 days later using G418. Positive clones were pooled and expanded.

### 2.8. RNA Isolation, Reverse Transcription and Real-Time Quantitative PCR

To quantify mRNA levels of cellular genes isolated from the above-mentioned human cells from monolayer culture and from fresh frozen OPSCC samples, quantitative reverse transcription-PCR (RT-qPCR) using the LightCycler system (Roche, Mannheim, Germany) was performed as previously described [24]. The primers used for this study were: VEGFA-fw: CCTCCGAAACCATGAACTTT; VEGFA-rev: TTCTTTGGTCTGCATTCACATT; VEGFR1-fw: TTTGGATGAGCAGTGTGAGC; VEGFR1-rev: CGGCACGTAGGTGATTTCTT; VEGFR2-fw: CTCTTGGCCGTGGTGCCTTTG; VEGFR2-rev: GTGTGTTGCTCCTTCTTTCAAC; HPRT1-fw: TGACACTGGCAAAACAATGCA; HPRT1-rev: GGTCCTTTTCACCAGCAAGCT.

### 2.9. Statistics

The sample size was determined before analysis with a power of 90% and a significance level for beta-error $\leq 0.05$, including $n = 35$ HPV-positive and $n = 21$ HPV-negative tumor samples. Clinicopathological features were analyzed using cross-tabulations, $\chi 2$ test and Fisher's exact probability test using SPSS 27 software (IBM, Armonk, NY, USA). The overall survival was calculated using the Kaplan–Meier algorithm for incomplete observations. Outcomes were measured from the time of diagnosis to the last day the patient was alive (censored data) or died for any reason (uncensored data). The log-rank (Mantel-Cox) test was used to perform a univariate analysis of the different variables. RT-qPCR data were analyzed with GraphPad Prism 8 (GraphPad Software, La Jolla California, USA) using ANOVA. The staining intensities of tumor cells, blood vessels or vessel density were analyzed using the Wilcoxon test for dependent non-normally distributed groups, the Mann–Whitney U test for independent non-normally distributed groups and the t-test for independent normally distributed groups as indicated. Results at a significance level of $p \leq 0.05$ in two-sided tests were considered statistically significant. All data from RT-qPCRs were expressed as mean ± SD. Statistical significance was determined with unpaired two-tailed Student's t-test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

## 3. Results

### 3.1. Characterization of VEGFR2 Expression in Blood Vessels of HPV-Positive and HPV-Negative OPSCC

Since ROS and OS were previously demonstrated to have a strong effect on the expression of VEGF/VEGFR2, we aimed at characterizing whether differences in the expression pattern of VEGFR2 exist in 56 OPSCC with known HPV-status and OS signatures [13,25]. We, therefore, determined VEGFR2 levels on blood vessels from tumor-free and tumor-containing regions. Furthermore, we aimed at detecting differences in the vessel density of HPV-positive versus HPV-negative OPSCC.

To prove that the VEGFR2-positive cells are blood vessel lining endothelial cells, we performed double immunofluorescence staining with the endothelial cell marker CD31 together with VEGFR2. In most capillaries of the HPV-positive samples, endothelial cells showed strong colocalization of CD31 with VEGFR2. In HPV-negative sections, however, a sparse colocalization of CD31-positive endothelial cells with VEGFR2 was observed (Figure 2A).

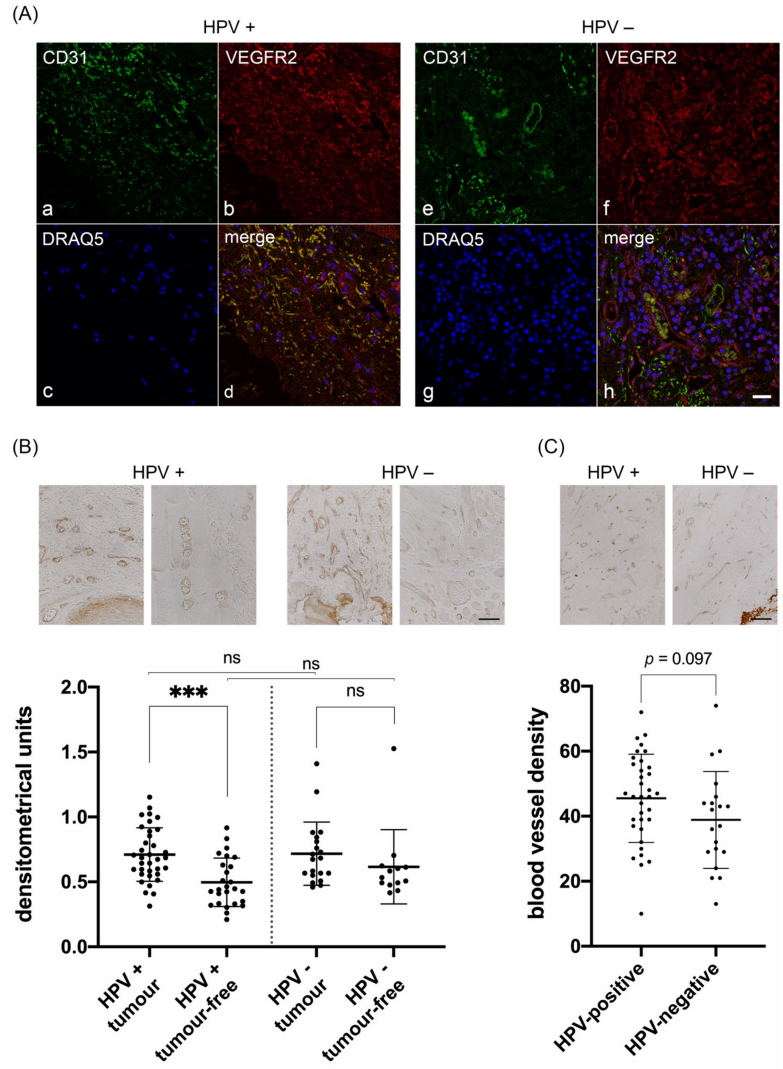


**Figure 2.** (**A**) Colocalization analysis of VEGFR2 with CD31 (endothelial cell marker) by immunofluorescence labelling of OPSCC. Antibody stainings were visualized by confocal microscopy. Colocalization of CD31 (**a**) with VEGFR2 (**b**) revealed that VEGFR2 is expressed in numerous capillaries of HPV-positive OPSCC (**d**). Colocalization of CD31 (**e**) with VEGFR2 (**f**) revealed that VEGFR2 is only occasionally present in capillaries of HPV-negative tumors (**h**). Cell nuclei were stained with DRAQ5 (**c,g**). Scale bar: A-H 20 µm. (**B**) Representative immunohistochemical staining and corresponding staining intensity analysis of VEGFR2 expression in blood vessels of tumor containing and adjacent tumor-free regions in (left) HPV-positive and (right) HPV-negative OPSCC. HPV-positive tumor regions (M = 0.657 DU; SD = 0.178 DU), adjacent tumor-free regions (M = 0.497; SD = 0.187), (***, $p < 0.0001$); HPV-negative tumor regions (M = 0.675; SD = 0.251), tumor-free regions (M = 0.616; SD = 0.286), (ns, $p = 0.107$); HPV-positive and -negative tumor regions (ns, $p = 0.740$); HPV-positive and-negative tumor-free regions (ns, $p = 0.129$). (**C**) Blood vessel density per viewing field of VEGFR2-immunoreactive capillaries in HPV-positive and HPV-negative OPSCC. Vascular density of HPV-positive (M = 45.51; SD = 13.574) and HPV-negative OPSCC (M = 38.85; SD = 14.901), (ns, $p = 0.097$). (DU = densitometrical units, M = mean, SD = standard deviation, ns = not significant, scale bars: A 50 µm; B 100 µm).

Next, we performed immunohistochemical staining to analyze differences in the expression level of VEGR2 in blood vessels related to the HPV-status of the tumor. In both HPV-positive and -negative tumors, blood vessels were positive for VEGFR2 immunostaining, however, with obvious differences in the staining intensities (Figure 2B).

We next determined the staining intensity of VEGFR2 by densitometrical analysis of representative viewing fields of three blood vessels each in tumor regions and tumor-free regions for comparison within the same sections (in densitometrical units (= DU)). Interestingly, in the subgroup of HPV-positive tumors, we observed significantly stronger staining intensities of blood vessels in tumor regions compared to tumor-free regions (Figure 2B, left; $p < 0.0001$). In contrast, in the HPV-negative group, the difference in staining intensities of blood vessels between tumor regions and tumor-free regions was not significant (Figure 2B, right; $p = 0.107$). Also, when comparing blood vessels of HPV-positive and -negative tumor or tumor-free regions, differences in VEGFR2 staining intensities were not significant either ($p = 0.740$ and $p = 0.129$, respectively).

To further analyze whether VEGFR2 mediated angiogenesis might be generally increased in HPV-positive compared to HPV-negative OPSCC, the number of VEGFR2-immunoreactive capillaries per viewing field was analyzed. However, comparing both groups, only a trend, but no significant increase in the number of vessels could be observed (Figure 2C; $p = 0.097$).

### *3.2. Analysis of VEGFR2 Staining Intensity in Tumor Cells of HPV-Positive and HPV-Negative OPSCC*

Since we observed that VEGFR2 expression was not only restricted to blood vessels but could also be detected within tumor cells, we additionally analyzed whether the HPV-status influences VEGFR2 immunoreactivity within tumors. Regardless of the HPV-status, we observed a general heterogeneity of staining intensities within each tumor section with areas of low, medium and high VEGFR2 expression.

To determine these expression differences, areas of low (median 0.2 DU (CI 0.13–0.23)), medium (median 0.49 DU (CI 0.38–0.63)) and high staining intensities (median 1.45 DU (CI 1.12–1.75)) were measured by densitometry for each tumor sample. In general, VEGFR2 expression was found to be higher in HPV-negative tumors. This difference was particularly significant for VEGFR2 expression levels at high-intensity (Figure 3C; $p = 0.012$) and at medium intensity (Figure 3B; $p = 0.014$). In regions with low VEGFR2 expression, this difference was not significant but again showed a trend toward higher expression in HPV-negative tumors (Figure 3A; $p = 0.140$).

In order to clarify whether the VEGFR1, VEGFR2 and VEGFA genes are transcriptional targets of HPV16 oncogenes, we quantified their mRNA expression levels by RT-qPCR in monolayer cultures of FaDu cells, primary tonsillar keratinocytes as well as primary foreskin keratinocytes. No strong effects could be measured in all keratinocyte types for VEGFR1 and VEGFA (Figure S5A–C). VEGFR2 could not be quantified at all in cell culture. However, in RNAs, isolated from fresh OPSCC samples, the mRNA expressions of VEGFR1 and VEGFR2 were significantly higher in HPV-positive samples than in HPV-negative samples. (Figure S5D).

To prove the keratinocytic origin of VEGFR2-positive tumor cells and to evaluate differences in the subcellular localization of VEGFR2, we performed double immunofluorescence analyses with antibodies against $p16^{INK4A}$ to detect HPV-positive tumor cells and TP53 to detect HPV-negative tumor cells. In both tumor entities, cytoplasmic and nuclear VEGFR2-immunoreactivity was detected. However, nuclear expression of VEGFR2 in HPV-positive OPSCC samples was found to be stronger (Figure 4A). Additionally, strong VEGFR2-positive cells, which were diffusely distributed in the lamina propria and around capillaries were also found in both tumor entities. However, these cells were detected more frequently in HPV-positive ($n = 16/35$) than in HPV-negative ($n = 7/21$) tumors.

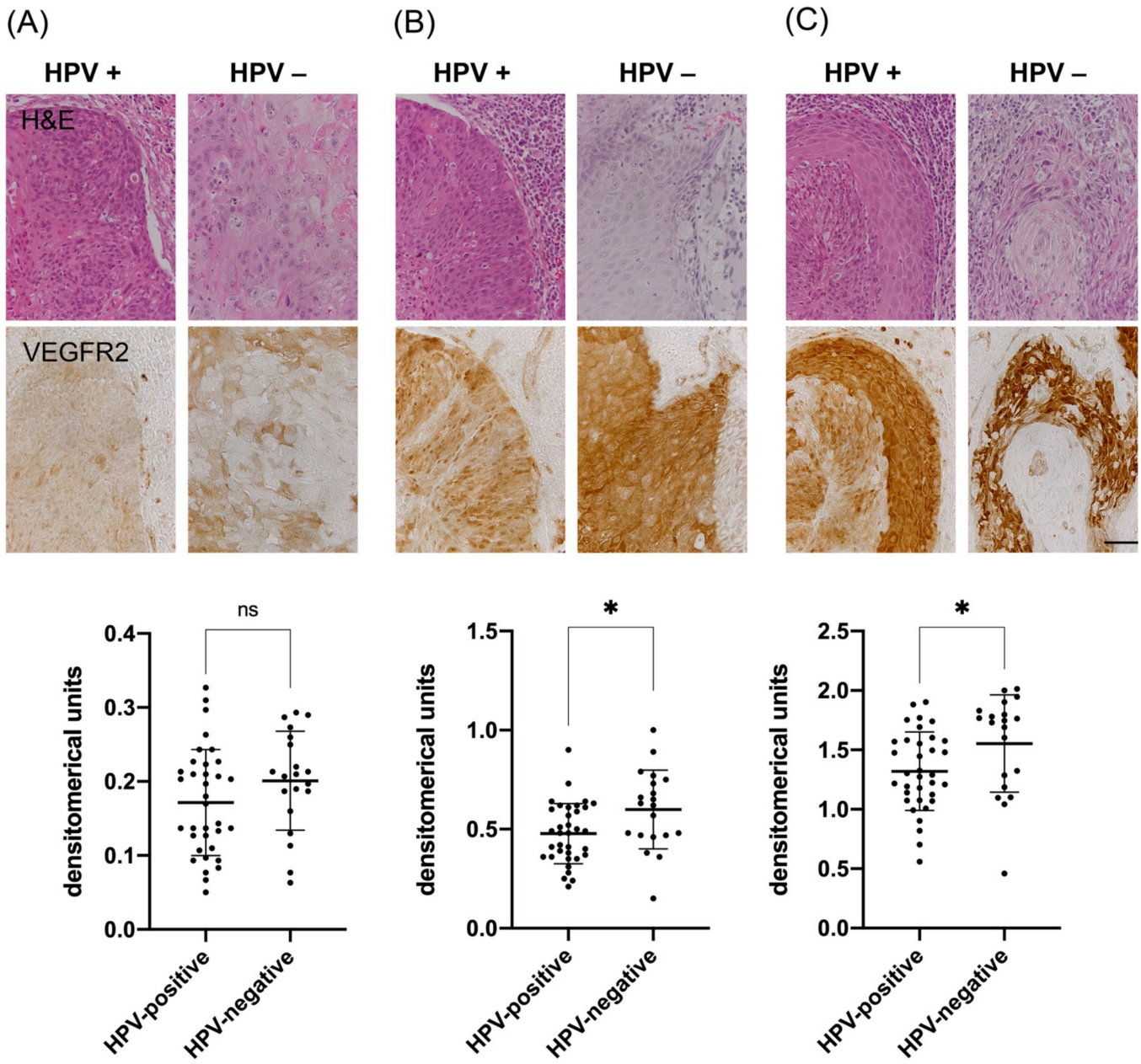


**Figure 3.** Analysis of VEGFR2 staining intensity in tumor cells of HPV-positive and HPV-negative OPSCC. Representative immunohistochemical VEGFR2 staining images (A = low, B = medium and C = high staining intensity), as well as corresponding H&E stain images of consecutive sections. (**A**) Staining intensities of low VEGFR2 expressing HPV-positive (M = 0.172 DU; SD = 0.072 DU) and HPV-negative (M = 0.201 DU; SD = 0.067 DU) tumor cells, $p = 0.140$. (**B**) Moderate expression levels of VEGFR2-positive tumor cells in HPV-positive (M = 0.478; SD = 0.152) vs. HPV-negative samples (M = 0.600; SD = 0.199), *, $p = 0.014$. (**C**) High expression levels of VEGFR2-positive tumor cells in HPV-negative (M = 1.714; SD = 0.260) and HPV-positive specimens (M = 1.382; SD = 0.320), *, $p = 0.012$. (DU = densitometrical units, M = mean, SD = standard deviation, ns = not significant, scale bar 50 μm).

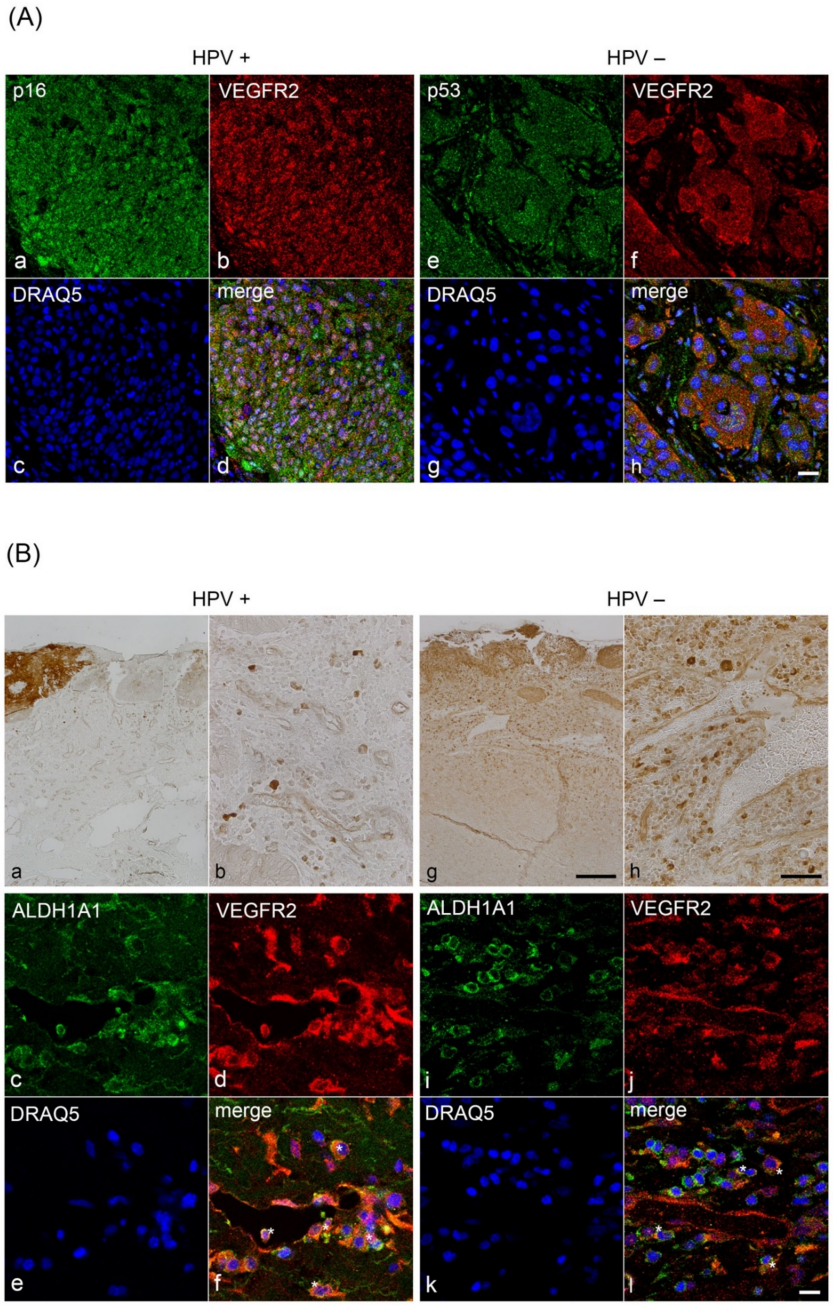


**Figure 4.** (**A**) Colocalization analysis of VEGFR2 with $p16^{INK4A}$ (tumor cell marker of HPV-positive tumors) and VEGFR2 with p53 (tumor cell marker of HPV-negative tumors) by immunofluorescence labeling of OPSCC. Antibody stainings were visualized by confocal microscopy. Colocalization of $p16^{INK4A}$ (**a**) with VEGFR2 (**b**) was detected in the cytoplasm and cell nuclei of HPV-positive tumor cells (**d**). Colocalization of p53 (**e**) with VEGFR2 (**f**) was identified in the cytoplasm and cell nuclei of HPV-negative tumor cells. Note, that VEGFR2 was detected in numerous tumor cell nuclei of HPV-positive OPSCC (**d**) compared to HPV-negative OPSCC (**h**). Tumor cell nuclei were stained with DRAQ5 (**c**,**g**). Scale bar: 20 µm. (**B**) Colocalization analysis of VEGFR2 with ALDH1A1 (CSC marker) by immunofluorescence labeling of OPSCC. Immunohistochemical staining against VEGFR2 of a representative consecutive HPV-positive (**a**–**f**) and HPV-negative tumor section (**g**–**l**). (**a**,**g**) Overview and (**b**,**h**) details. A subpopulation of tumor cells with strong VEGFR2 immunoreactivity can be observed. Colocalization of VEGFR2 (**d**,**j**) with ALDH1A1 (**f**,**l**) was detected only in a subpopulation of tumor cells and at the subcellular level mainly in the cytoplasm (asterisks). The cells were distributed around the blood vessels (**f**,**l**). In HPV-positive OPSCC, some migrating cells were detected at the blood vessel wall and one cell is visible intravasally, while others were recognized in the fibrous tissue (**f**). Cell nuclei were stained with DRAQ5 (**e**,**k**). Scale bars: (**a**,**g**) 200 µm; (**b**,**h**) 50 µm; (**c**–**f**), (**i**–**l**) 20 µm.

Based on the fact that VEGFR2 positive cells were localized at sites typical for the localization of cancer stem cells (CSCs), we examined whether these cells might show CSCs characteristics [6]. Therefore, we performed double immunofluorescence staining with VEGFR2 and the CSC-marker ALDH1A1 [26]. Distinct cells showed colocalization of VEGFR2 with ALDH1A1, which was paralleled by high cytoplasmic ALDH1A1 expression levels. Cells with a distinct colocalization pattern were predominantly found next to blood vessels. These colocalization signals were frequently observed in HPV-positive OPSCC, however, we sparsely detected them in HPV-negative OPSCC (Figure 4B).

### *3.3. Correlation of VEGFR2, NRF2 and AKR1C3 with Clinicopathological Data*

We recently demonstrated that the oxidative stress markers NRF2 and AKR1C3 are overexpressed in both subgroups of HPV-positive and HPV-negative OPSCC and correlate with unfavorable survival [15]. As ROS and OS were demonstrated to have a strong effect on the expression of VEGF/VEGFR2, we performed immunohistochemical staining against NRF2 and AKR1C3 to prove the occurrence of OS in the tumor tissue (Figure S4) [13,25]. By correlating the immunohistochemical results with clinicopathological data, we demonstrated a highly significant correlation of $NRF2^{high}$ and $AKR1C3^{high}$ tumors with worse overall survival (OS) ($p < 0.0001$ and $p < 0.001$, respectively) (Table 1 and Table S1). We also separately correlated VEGFR2, NRF2 and AKR1C3 staining in HPV-positive and HPV-negative tumors in relation to survival. These analyses revealed that particularly high VEGFR2 expression in HPV-positive tumors is associated with an unfavorable prognosis ($p = 0.013$) (Figure S6 and Table S1). High T-stage correlated with worse OS ($p = 0.049$) in all cases (Table S1). However, T-stage did not reach significance in separate correlations of both HPV-positive and-negative subgroups (Figure S7). There was a strong association for VEGFR2 protein expression with HPV-status (Table 1). Furthermore, HPV-status was associated with parameters such as a higher blood vessel density and a higher proportion of non-smokers to smokers (Table 1).

## 4. Discussion

Autocrine VEGF signaling can contribute to tumor initiation and progression and is regulated by the receptor tyrosine kinase VEGFR2 in several tumors including HNSCC [6,10]. Although it is known that VEGFR2 is generally overexpressed in blood vessels supplying HNSCC, it remains to be determined whether HPV exerts specific effects on VEGFR2 expression and thus possibly on the regulation of vascularization [9,10]. Studies on cervical tumors and in vitro studies using HPV-positive cervical cancer cell lines indicated such a possibility [11,12,27]. It is speculated that HPV generally contributes to the vascularization of tissues by upregulating VEGF/VEGFR signaling to support the high energy demands of infected cells, which in turn also promotes tumor growth and malignant transformation [27,28]. In addition, a variety of tumor cells, including HNSCC, are known to express VEGF and VEGFR2 at high levels so that they can promote tumor growth, invasion and survival by autocrine signaling responses [6,10,29]. However, previous studies on HNSCC have not focused specifically on OPSCC and HPV-status has not been considered [9].

We, therefore, analyzed the expression patterns of VEGFR2 both in blood vessels of tumor regions and tumor-free regions and in tumor cells of HPV-positive and -negative OPSCC by means of densitometric analysis. Since oxidative stress (OS) is known to induce VEGF expression and we previously showed that both HPV-positive and -negative OPSCC present with subgroups overexpressing OS signatures going along with poor prognosis, NRF2 expression and analysis of its target gene AKR1C3 serving as a read-out for activated OS signatures was included in this study [30,31]. Differences in VEGFR2 expression between HPV-positive and -negative OPSCC were detectable in both blood vessels and tumor tissue, suggesting that depending on HPV status, VEGFR2 signaling plays a crucial role in OPSCC progression.

Our results strongly imply that VEGFR2 may be upregulated in endothelial cells of blood vessels supplying nutrients to HPV-positive tumor regions and that this is not the case in HPV-negative OPSCC. Therefore, our observation might suggest that angiogenesis may be upregulated under the influence of HPV by regulating the expression of VEGFR2 in blood vessels. However, further studies have to clarify by which mechanisms HPV-positive tumor cells might regulate increased VEGFR2 expression in surrounding tumor blood vessels.

This hypothesis is furthermore supported by the observation that HPV-positive OPSCC tends to have a higher density of VEGFR2-expressing blood vessels compared to HPV-negative tumors (Figure 2). While this analysis did not reach significance, the absolute number of tumors with a high density of VEGFR2-expressing blood vessels, however, proved to be significantly higher (Table 1). This key observation is supported by xenograft models in which HPV-positive and -negative cells were incubated under hypoxic conditions and then applied to nude mice [32]. Those mice that received HPV-positive tumor cells showed a higher density of neo-blood vessels, which resulted in improved blood supply and thus less hypoxic tumor areas, which was paralleled by lower mRNA expression of hypoxia-responsive genes such as HIF-1$\alpha$, GLUT-1 and VEGF-A. Moreover, in that study, tumor samples were analyzed by immunohistochemical detection of neo-blood vessels using the vascular endothelial cell proliferation marker CD105. This led to the observation of HPV-positive OPSCC having higher numbers of blood vessels.

We, therefore, considered analyzing the activation status of VEGFR2 to detect only angiogenically active blood vessels by immunohistochemical staining with antibodies directed against phosphorylated VEGFR2 at Tyr 951, as this modification plays a significant role in tumor angiogenesis and growth [33]. However, we could not detect specific immunoreactivity using the only available antibody suitable for immunohistochemistry thus far (monoclonal Phospho-VEGFR2 (Tyr951) (15D2); #4991 Cell Signaling; data not shown).

The expression of CD31 in endothelial cells modulates cell adhesion, endothelial cell migration and angiogenesis [30]. However, CD31 only gives a static representation of vessel density, whereas VEGFR2 expression may better reflect the physiological stimulus for endothelial growth [31]. In HPV-negative tumors, VEGFR2 and CD31 only partially colocalized in the endothelium of blood vessels and capillaries. The strong colocalization of CD31 and VEGFR2 in blood vessels and capillaries of HPV-positive OPSCC, however, indicates that HPV induces a highly upregulated angiogenic activity, pointing to significant differences in angiogenesis based on HPV-status [34,35]. This upregulated angiogenic activity may result in an improved response to radiochemotherapy, as individual tumor cells may be more accessible by the bloodstream, leading to a more favorable prognosis compared to HPV-negative OPSCC [36,37]. Mechanistically, increased perfusion delivers oxygen that promotes ROS/free radicals essential for the induction of radiation-induced DNA damage upon radiotherapy thus making cells more accessible for the influx of chemotherapeutic agents.

On the other hand, an improved radiation response may also be achieved by blocking VEGFR2 through anti-VEGF therapy [38,39]. Typically, epithelial tumors can respond to radiotherapy with growth factor-driven revascularization, including increased VEGFR2 expression, which may be prevented by anti-VEGF therapy that inhibits revascularization. This, in turn, would lead to increased blood flow and thus a better oxygen supply, resulting in increased ROS formation during radiation therapy and increased flooding of the chemotherapy to the already existing tumor cells. Due to the lack of new blood vessel formation, on the other hand, the formation of new tumor tissue is prevented [37–39]. In this study, upregulation of VEGFR2 and the OS marker AKR1C3 was associated with an unfavorable prognosis in HPV-positive OPSCC.

In addition, we could show that VEGFR2 is also expressed in tumor cells. Both HPV-positive and -negative OPSCC presented with a heterogeneous expression pattern including low, moderate and high VEGFR2-immunoreactivity. This may suggest that aberrant VEGFR2 expression in tumor cells, together with its downstream signaling pathways,

may be involved in other besides angiogenesis. However, mutations in VEGFR2 do not seem to be of relevance, as they only show low alteration rates in HNSCC (2.3% in the TCGA cohort, cBioPortal, data not shown [40,41]). Therefore, other mechanisms like varying differentiation states of tumor cells within one tumor, or areas that have a need for improved nutrients and oxygen supply compared to other regions, might correlate with increased VEGFR2 expression levels.

Densitometric analysis showed a significantly higher expression of VEGFR2 in HPV-negative tumor cells. This may suggest that the differential expression of VEGFR2 between HPV-positive and -negative tumor cells exhibit crucial cell biological differences.

Several oncogenes such as the epidermal growth factor receptor (EGFR) and oncogenic transcription factors such as c-myc are capable of upregulating VEGF expression. Furthermore, wild-type TP53 indirectly represses VEGF [42]. However, HPV16-E6 seems to induce VEGF expression independently of TP53 inactivation, using the SP1 transcription factor for E6-mediated induction of the VEGF promoter [11]. This could also be supported by our observation that VEGFR2 was frequently translocated into the nuclei of HPV-positive but less frequently into the nuclei of HPV-negative tumor cells. It is known that VEGFR2 is translocated to the nucleus of neoplastic cells upon phosphorylation and that VEGFR2 therein may interact with transcription factors such as SP1 to regulate gene transcription [43,44]. Biologically, this might be a self-enhancing mechanism in response to hypoxia and/or OS. In vitro studies using HeLa cells showed that the fraction of nuclear-positive cells increased due to hypoxic stimulation [43].

Degradation of VEGFR2 is furthermore mediated by the recruitment of the E3 ubiquitin ligase β-Trcp1 followed by polyubiquitination and delivery to the proteasome [45]. β-Trcp1 is upregulated by HPV16-E7 expression in vitro [46]. Moreover, β-Trcp1 is also involved in the regulation of HIF1α the WNT/β-Catenin and the PI3K/AKT pathway [47,48]. β-Trcp1 also provides an alternative way to regulate the NRF2/OS pathway for proteasomal degradation via Keap1/Cul3 [49]. Therefore, additional side effects may exist, especially considering that all these signaling pathways often present with alterations especially in HPV-negative OPSCC.

Noteworthily, a subpopulation of tumor cells presented with high VEGFR2 expression levels. Based on their localization in a perivascular niche adjacent to endothelial cells, it may be suggested that these cells are CSCs. It is known that the amount of this stem cell population and their self-renewal can be regulated by autocrine VEGF/VEGFR2 signaling [6,50]. Double immunostaining with the tumor stem cell marker ALDH1A1 maintaining CSC properties indeed showed colocalization of VEGFR2 with ALDH1A1. This suggests that these cells might be CSCs localized in the proposed perivascular niche [26].

In line with studies analyzing CSCs in the comparison of HPV-positive and -negative OPSCC, we observed a higher number of ALDH1A1$^+$/VEGFR2$^+$ in HPV-positive OPSCC [51,52]. We recently showed that HPV16 is capable of modifying the phenotype of infected CSCs by increasing the pool of migratory CSCs through the expression of HPV16-E6E7 in vitro and in HPV-positive OPSCC [22]. Furthermore, autocrine VEGF/VEGFR2 signaling enhances tumor invasion and survival by promoting processes crucial for CSCs like dedifferentiation and an epithelial-mesenchymal transition phenotype [6]. Furthermore, VEGFR2 can regulate epithelial tumor stem cell migration [53]. Taken together, the higher number of ALDH1A1 and VEGFR2 colocalizing tumor cells in HPV-positive OPSCC might indicate that these tumors are associated with a higher number of migratory CSCs.

## 5. Conclusions

To analyze VEGFR2 expression patterns in HPV-positive and-negative OPSCC, we performed quantitative immunohistochemical and immunofluorescence labeling of formalin-fixed, paraffin-embedded (FFPE) samples with known HPV-status. VEGFR2-staining intensities in blood vessels of tumor-containing and tumor-free regions, as well as in tumor cells, were quantified and compared between HPV-associated and HPV-negative OPSCC.

In conclusion, we identified a distinct molecular protein expression profile of VEGFR2 in HPV-positive and HPV-negative OPSCC. In the HPV-positive group, we observed significant differences in VEGFR2 expression levels between blood vessels of tumor regions compared to tumor-free regions. In contrast, HPV-negative OPSCC presented with significantly higher VEGFR2 expression levels in tumor cells. Based on this observation, two different HPV-status-dependent phenotypes of VEGFR2 signaling may exist, possibly triggered by hypoxia and/or oxidative stress (Figure 5). Future studies should focus on unraveling the molecular basis of mechanisms involved in the differential regulation of VEGFR2 expression between HPV-positive and -negative OPSCC in tumor-supporting blood vessels and tumor cells. Such studies could prove to be of pivotal importance for the patient outcome when anti-VEGF therapies in the treatment of OPSCC are considered.

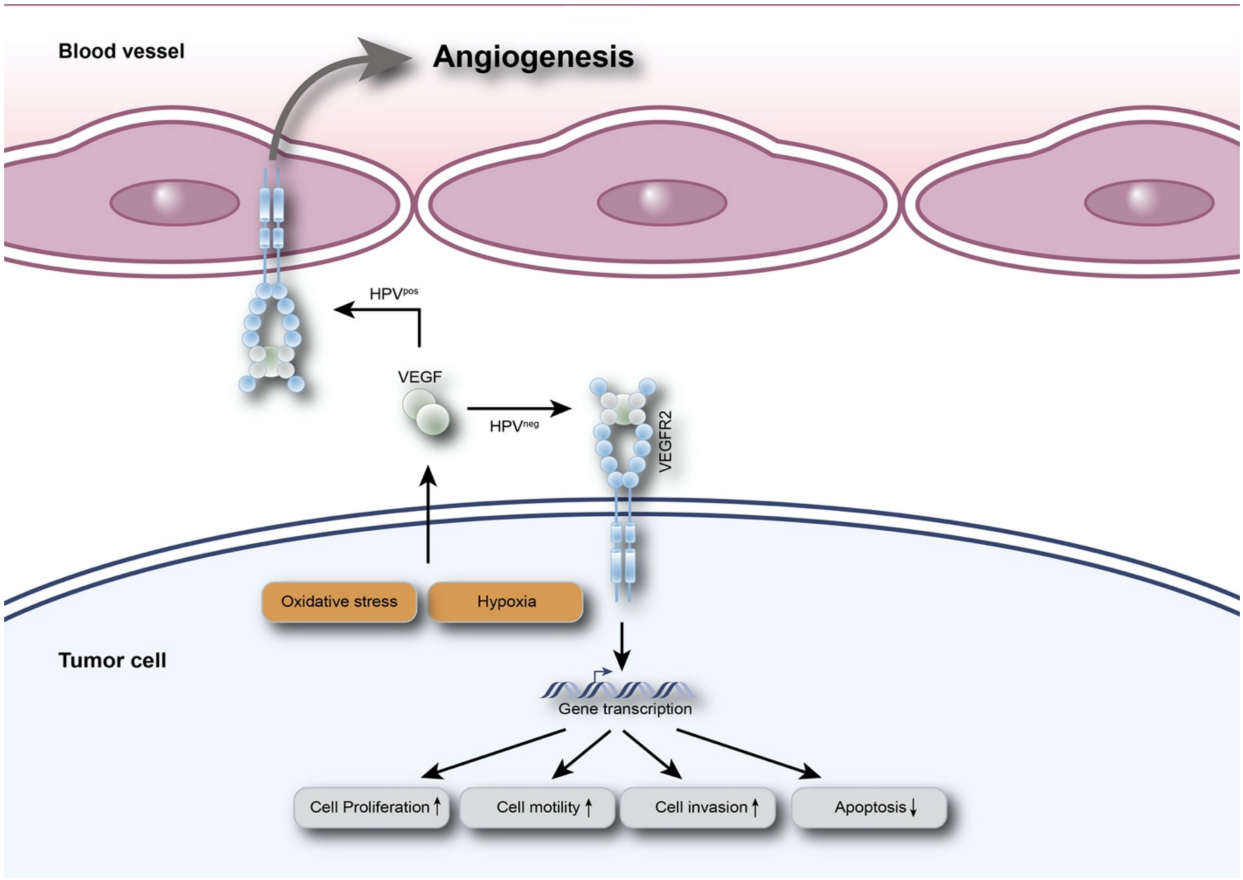


**Figure 5.** Schematic presentation of the two suggested HPV-status-dependent VEGFR2 signal pathways in OPSCC. Depending on HPV and possibly triggered by hypoxia and/or oxidative stress, VEGFR2 expression is upregulated in tumor blood vessels of HPV-positive OPSCC, which may be associated with increased angiogenesis. This is not observed in HPV-negative OPSCC; instead, VEGFR2 is significantly stronger expressed in the tumor cells themselves, which may lead to increased activation of tumor cell proliferation, migration, invasion and reduced apoptosis. The increased tumor cell activity may also be correlated to the tendency of lower blood vessel count in HPV-negative OPSCC due to hypoxia. Figure modified from [10].

Furthermore, our data indicate that VEGFR2 may play a regulatory role in CSCs of HPV-positive OPSCC. CSCs are thought to be responsible for treatment failure in anti-cancer therapy. Experimental validation of the regulatory role of VEGFR2 in stem cell migration and dedifferentiation, especially in CSCs of HPV-positive OPSCC, will further contribute to the understanding of unfavorable development of (distant) metastases and recurrence.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/cancers13205221/s1, Supplementary Information 1, Figure S1: Immunohistochemical positive controls of VEGFR2 expression in tissue samples of liver, cervix squamous cell carcinoma, melanoma, papillary thyroid carcinoma Figure S2: Immunohistochemical controls of the secondary antibodies and the detection system, Figure S3: Control of the confocal double immunofluorescence detection system, Figure S4: Representative immunohistochemical stainings of NRF2 and AKR1C3 in tumor tissue samples, Figure S5: MRNA expression of VEGFA, VEGFR1 and VEGFR2 were measured using reverse transcribed total cellular RNA from FaDu cells, primary tonsil keratinocytes, primary foreskin keratinocytes and HPV-negative or -positive OPSCC by RT-qPCR and normalized to HPRT1 mRNA levels, Figure S6: Univariate survival analysis for VEGFR2, NRF2 and AKR1C3 expression-status separated by HPV-status, Figure S7: Univariate survival analysis for T-stage and N-stage separated by HPV-status, Table S1: Univariate survival analysis.

**Author Contributions:** Conceptualization, S.U., Y.K. and C.U.H.; validation, S.U., Y.K. and C.U.H.; formal analysis, S.U., C.U.H. and M.H.; investigation, S.U., O.G.S., H.G.D. and C.U.H.; resources, Y.K., N.W., C.A., B.P., M.H., B.A. and J.P.K.; writing—original draft preparation, S.U. and M.H.; writing—review and editing, C.U.H., S.U., Y.K., N.W., C.A., B.P. and M.H.; visualization, S.U., C.U.H. and M.H.; supervision, J.P.K.; project administration, S.U., C.U.H. and Y.K.; funding acquisition, C.U.H., M.H. and H.G.D. All authors have read and agreed to the published version of the manuscript.

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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 on request from the corresponding authors. The data are not publicly available due to ethical restrictions.

**Conflicts of Interest:** All other authors declare no conflict of interest.

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**Supplementary Material**

*Supplementary Information*

Antigen retrieval of the deparaffinized sections was performed by heating at 95°C in 1 mM EDTA buffer (pH 8.0) for 15 min. After treatment with 0.25%-Triton-X 100 detergent solution, the sections were incubated with blocking solution containing 5% normal goat serum (NGS; Vector, Burlingame, USA) for 30 minutes. The sections were incubated with the appropriate first primary antibody (guinea-pig monoclonal anti-human CD31 (1:800; gift from Prof. Dr. M. Koch, Cologne), mouse anti-human polyclonal p16 (1:50; BD Biosciences, Heidelberg, Germany), mouse anti-human polyclonal p53 (1:25; biologo, Kronshagen, Germany) and mouse anti-human ALDH1A1 (1:500; sc-374076, sc-374149 Santa Cruz Biotechnology Inc., California, USA), overnight at 4°C. (Since the established antibody was no longer available, two new clones that showed the same results were evaluated.) Subsequently, the sections were incubated with the corresponding first secondary antibody (biotinylated goat anti-guinea-pig IgG (1:500; Vector), biotinylated goat anti-mouse IgG (p16 1:30, p53 1:20, ALDH1A1 1:300; Vector) for 60 minutes. Then these were incubated with 488-conjugated-NeutrAvidin (1:1000; Thermo Scientific, Massachusetts, USA) for 60 minutes. After treatment with 5% NGS blocking solution, the incubation with the second primary antibody rabbit anti-human VEGFR2 (1:500, ALDH1A1 1:200; 55B11 Cell Signalling Technology, Frankfurt am Main, Germany) was carried out overnight at 4°C. Afterwards, the sections were incubated with the second secondary antibody DyLight-550-conjugated goat anti-rabbit IgG (1:300, ALDH1A1 1:100; Thermo Scientific) for 60 minutes. The sections were incubated with the chromatin marker DRAQ5 (1:2000; Cell Signaling Technology) for 15 min in the dark to identify the cell nuclei and covered with Aqua-Poly/Mount (Polysciences, Hirschberg an der Bergstraße, Germany).

*Supplementary figures*

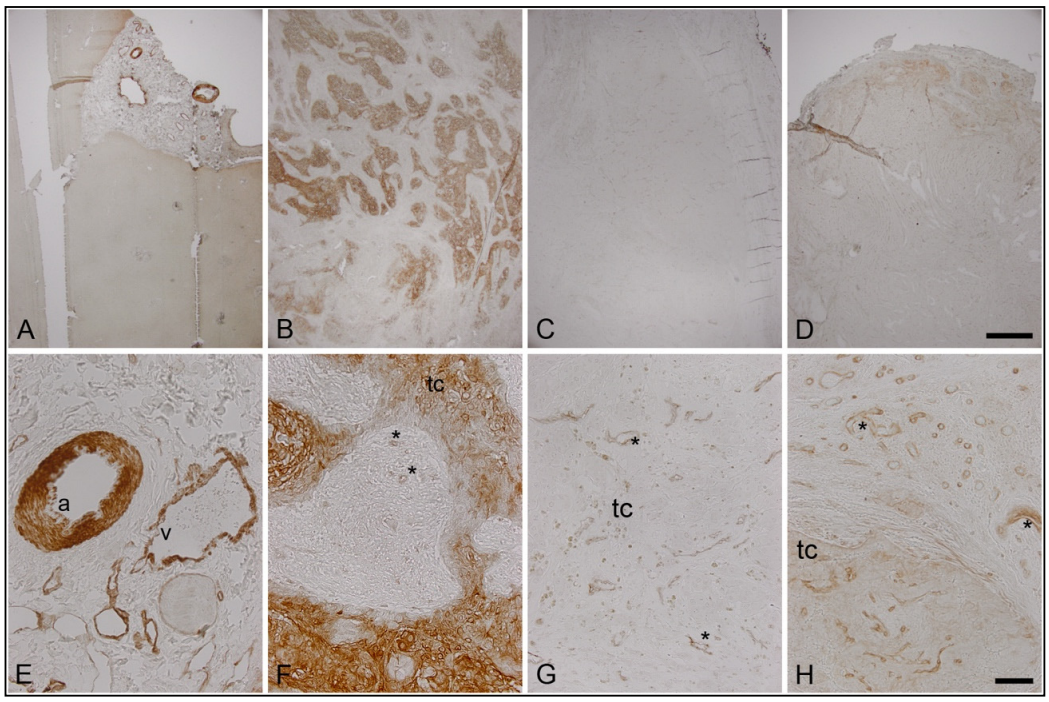


**Figure S1.** Immunohistochemical positive controls of VEGFR2 expression in tissue samples of liver, cervix squamous cell carcinoma, melanoma, papillary thyroid carcinoma. (**A, E**) Immunohistochemical staining against VEGFR2 of a positive control tissue section of liver to test the localisation of VEGFR2 in blood vessels. (**A**) Overview and (**E**) details. VEGFR2 is detected in arteries (**a**) and veins (**v**). (**B–H**) Immunohistochemical staining against VEGFR2 of positive control tumour tissue sections of cervix squamous carcinoma, melanoma and papillary thyroid carcinoma. (**B–D**) Overview and (**F–H**)

details). VEGFR2 is detected in blood vessels (asterisk) and tumour cells (**tc**) of cervix squamous cell carcinoma (**B, F**), melanoma (**C, G**) and papillary thyroid carcinoma (**D, H**). a = artery, v = vein, tc = tumour cells. Scale bars: A–D 1 mm, E-F 100 μm.

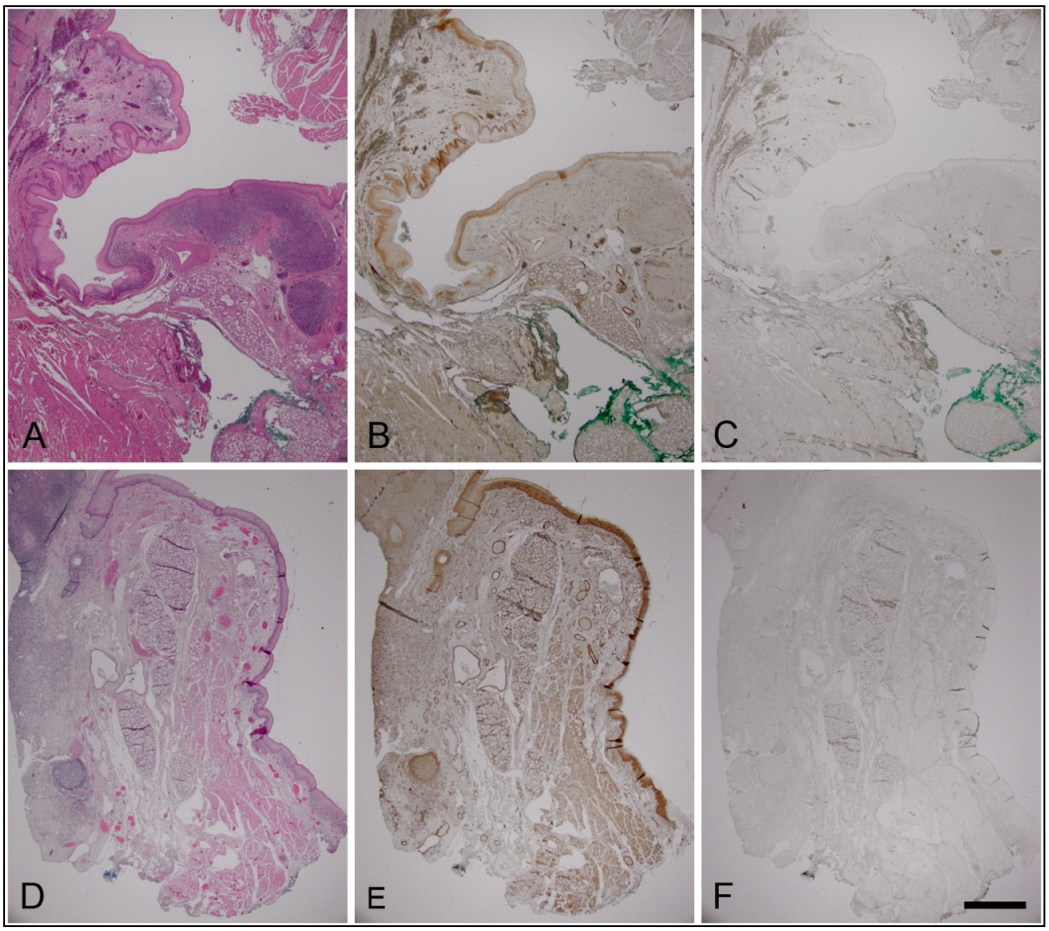


**Figure S2.** Immunohistochemical controls of the secondary antibodies and the detection system. (**A–C**) Consecutive slides of a representative HPV-positive and (**D–F**) HPV-negative OPSCC. (**A, D**) Histopathological characterisation by H&E staining, (**B, E**) immunohistochemical staining against VEGFR2, (**C, F**) control section incubated without VEGFR2 antibody. In comparison to the VEGFR2-immunoreactive sections (**B, E**), no immunohistochemical localisation is detected in the control section without primary antibody (**C, F**). Scale bar: 1 mm.

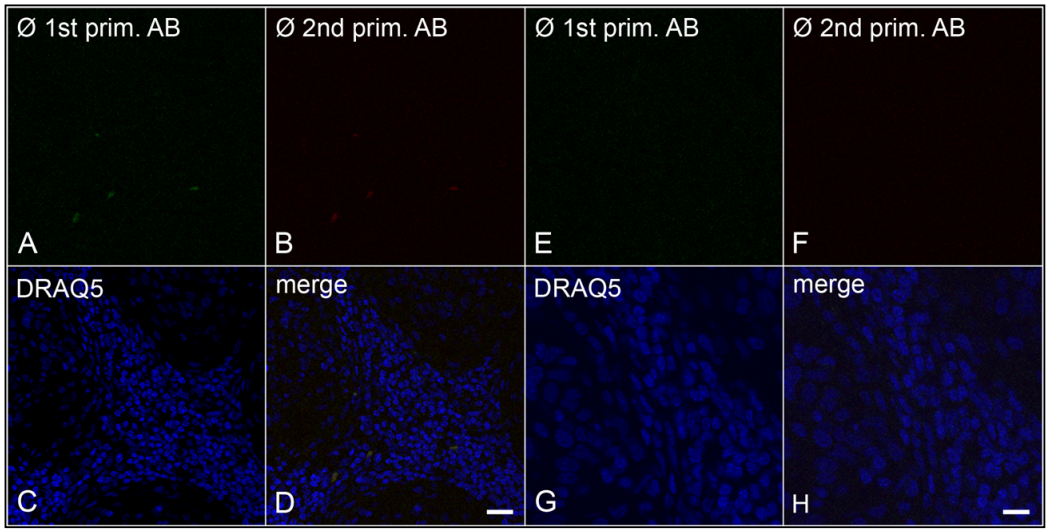

**Figure S3.** Control of the confocal double immunofluorescence detection system. (**A–D**) Overview and (**E–F**) details. In double immunofluorescence control incubations without first (**A, E**) and second primary antibodies (**B, F**), no specific staining is detectable (**D, H**). Tumour cell nuclei are stained with DRAQ5 (**C, G**). Single erythrocytes within blood vessels show autofluorescence (**D**). AB = antibody, ∅ = without. Scale bars: 20 µm.

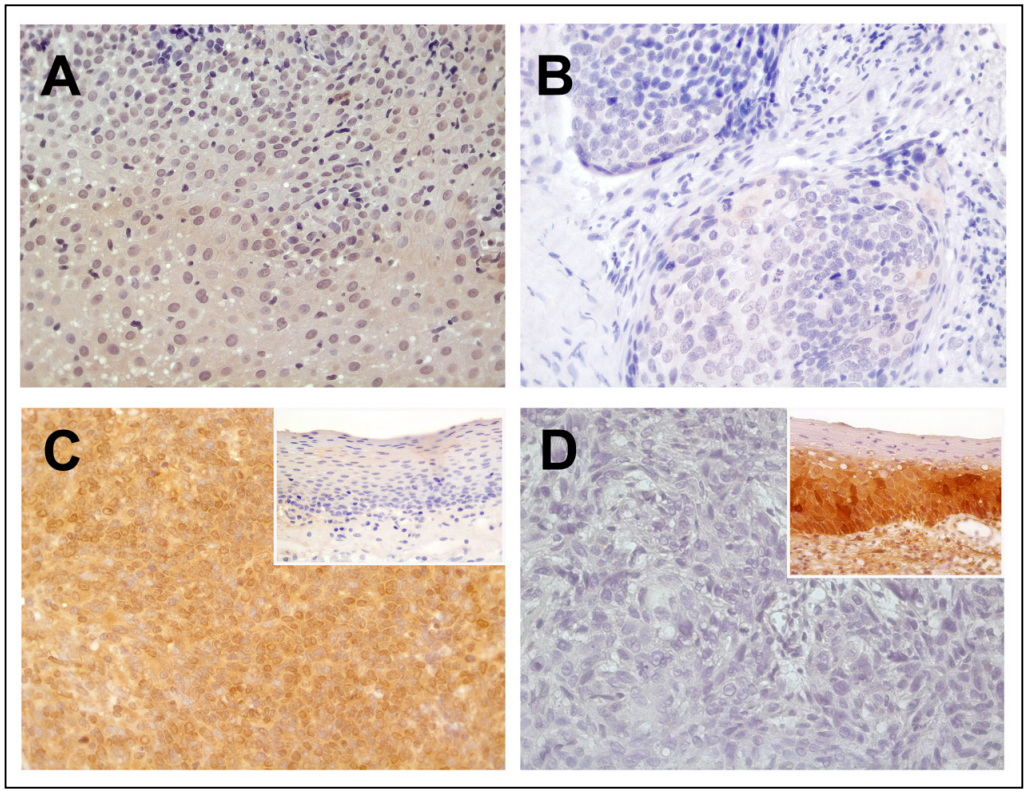


**Figure S4.** Representative immunohistochemical stainings of NRF2 (**A, B**) and AKR1C3 (**C, D**) in tumour tissue samples. (**A**) Tumour cells with positive nuclear staining against NRF2. (**B**) Lack of NRF2-staining. (**C**) AKR1C3-positive tumour tissue. (**D**) Negative AKR1C3-staining. Insets: adjacent normal squamous epithelium. *V* = x400.

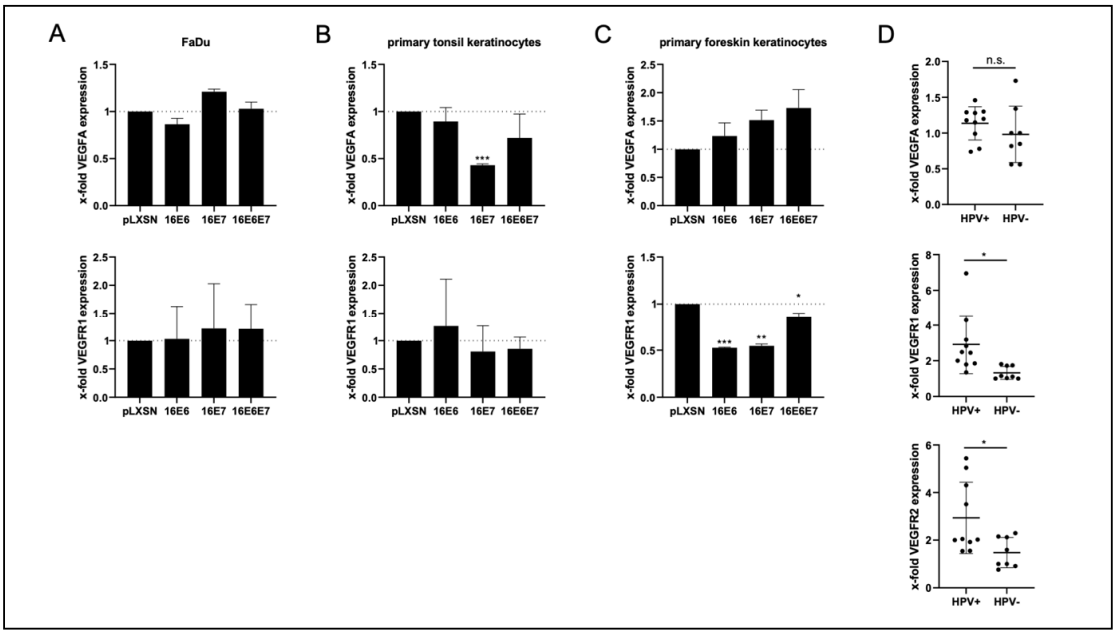

**Figure S5.** MRNA expression of VEGFA, VEGFR1 and VEGFR2 were measured using reverse transcribed total cellular RNA from (**A**) FaDu cells, (**B**) primary tonsil keratinocytes, (**C**) primary foreskin keratinocytes and (**D**) HPV-negative or -positive OPSCC by RT-qPCR and normalized to HPRT1 mRNA levels ($n$ = 3 independent experiments performed in duplicate). Error bars represent standard deviations. *, $p < 0.01$; **,$p < 0.001$; ***, $p < 0.0001$; n.s., No significant difference.

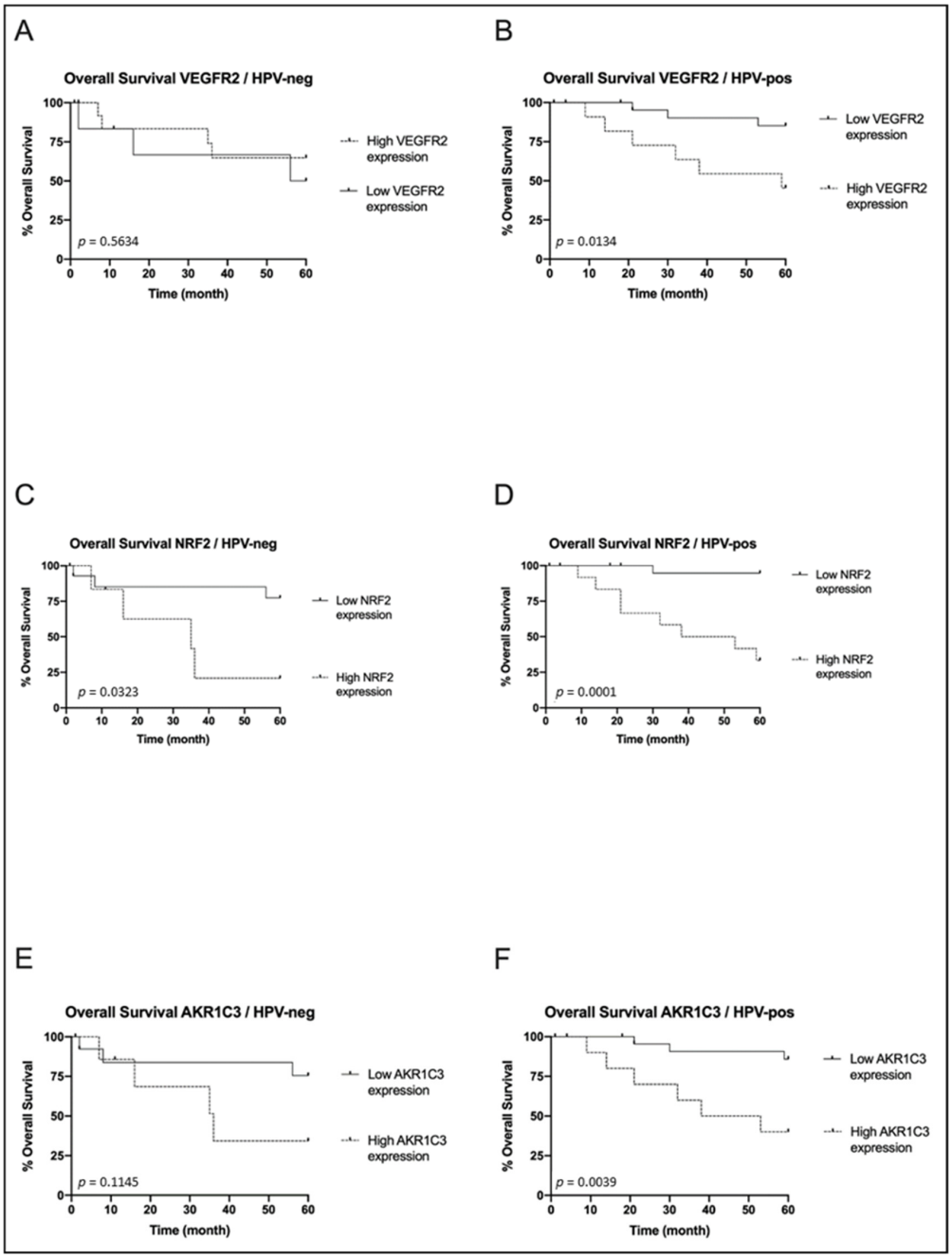


**Figure S6.** Univariate survival analysis for VEGFR2 (**A, B**), NRF2 (**C, D**) and AKR1C3 (**E, F**) expression-status separated by HPV-status. Kaplan–Meier plots for overall survival (OS) in patients with low vs. high protein expression. *p*-value was derived by log-rank/Mantel-Cox test.

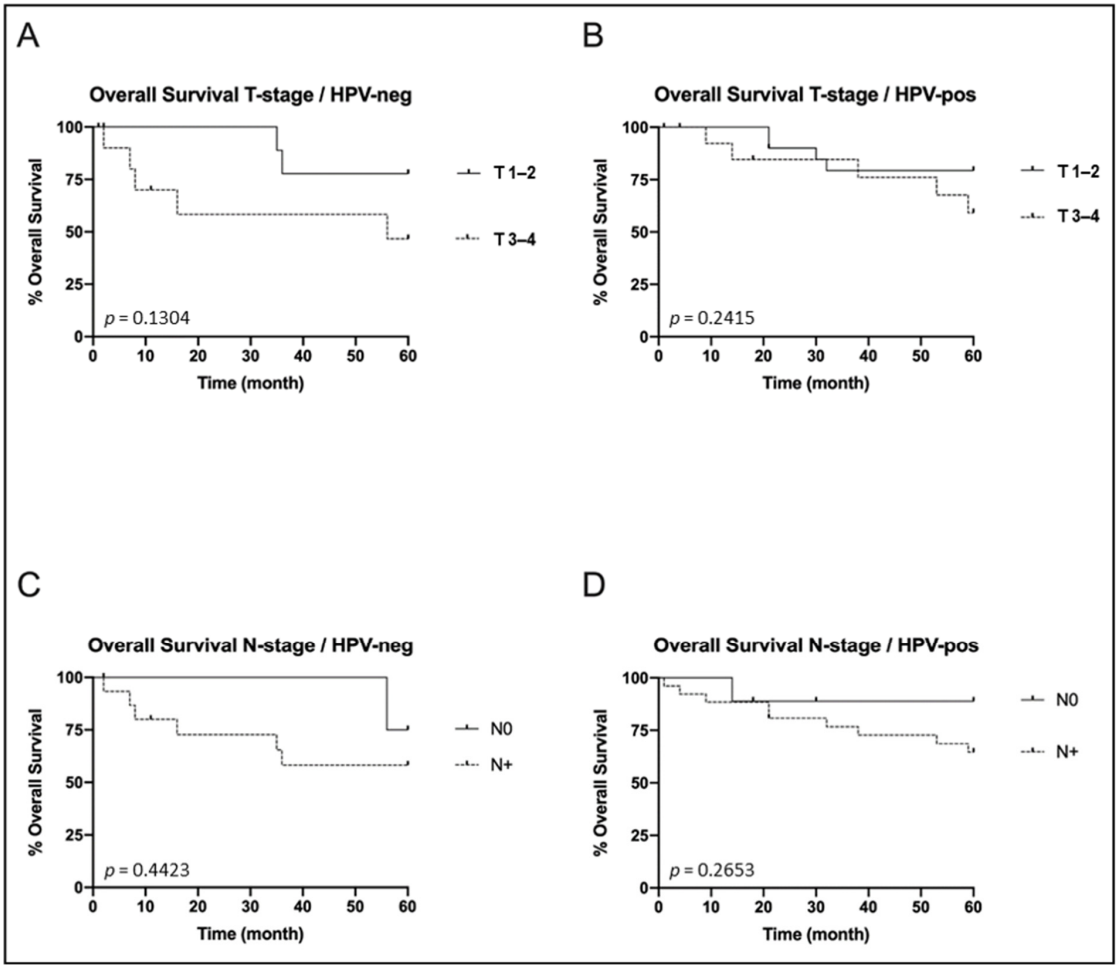


**Figure S7.** Univariate survival analysis for T-stage (**A, B**) and N-stage (**C, D**) separated by HPV-status. Kaplan–Meier plots for overall survival (OS) in patients with T 1–2 *vs.* T 3–4 and N0 *vs.* N+. *p* value was derived by log-rank/Mantel-Cox test.

*Supplementary table*

**Table S1.** Univariate survival analysis.

| Samples analysed | Parameters | Group | No. | Overall Survival (OS) | | |
|---|---|---|---|---|---|---|
| | | | | Hazard ratio | 95% CI | *p*-Value* |
| **Complete collection** | | | | | | |
| | All | | 55 | | | |
| | T stage | T1–2 | 26 | 0.4 | 0.1–1.0 | **0.049** |
| | | T3–4 | 29 | | | |
| | N stage | N0 | 41 | 0.5 | 0.2–1.4 | 0.176 |
| | | N+ | 14 | | | |
| | VEGFR2 | VEGFR2 low | 25 | 0.4 | 0.2–1.1 | 0.091 |
| | | VEGFR2 high | 30 | | | |
| | NRF2 | NRF2 low | 19 | 0.1 | 0.1–0.3 | **< 0.0001** |
| | | NRF2 high | 37 | | | |
| | AKR1C3 | AKR1C3- | 18 | 0.2 | 0.1–0.5 | **0.001** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | AKR1C3+ | 38 | | | |
| | | | | | | |
| **HPV-positive** | | | | | | |
| | T stage | HPV+/T1–2 | 20 | 0.433 | 0.11–1.7 | 0.242 |
| | | HPV+/T3–4 | 15 | | | |
| | | | | | | |
| | N stage | HPV+/N0 | 9 | 0.433 | 0.11–1.9 | 0.265 |
| | | HPV+/N+ | 26 | | | |
| | | | | | | |
| | VEGFR2 | HPV+/VEGFR2 low | 23 | 0.162 | 0.04–0.69 | **0.013** |
| | | HPV+/VEGFR2 high | 12 | | | |
| | | | | | | |
| | NRF2 | HPV+/NRF2 low | 23 | 0.059 | 0.01–0.2 | **< 0.0001** |
| | | HPV+/NRF2 high | 12 | | | |
| | | | | | | |
| | AKR1C3 | HPV+/AKR1C3- | 25 | 0.107 | 0.02–0.5 | **0.004** |
| | | | | | | |
| **HPV-negative** | | | | | | |
| | T stage | HPV-/T1–2 | 10 | 0.3 | 0.1–1.4 | 0.130 |
| | | HPV-/T3–4 | 11 | | | |
| | | | | | | |
| | N stage | HPV-/N0 | 5 | 0.5 | 0.1–2.8 | 0.442 |
| | | HPV-/N+ | 15 | | | |
| | | | | | | |
| | VEGFR2 | HPV-/VEGFR2 low | 7 | 1.6 | 0.3–7.8 | 0.563 |
| | | HPV-/VEGFR2 high | 13 | | | |
| | | | | | | |
| | NRF2 | HPV-/NRF2 low | 14 | 0.1 | 0.1–0.8 | **0.032** |
| | | HPV-/NRF2 high | | | | |
| | | | | | | |
| | AKR1C3 | HPV-/AKR1C3- | 13 | 0.3 | 0.1–1.4 | 0.115 |
| | | HPV-/AKR1C3+ | 8 | | | |

* *p*-Value calculated by log-rank (Mantel-Cox)test. Bold: significant values ≤ 0.050.

# 4. Discussion

In vitro studies using HPV-positive cervical cell lines and studies on cervical tumours indicate that HPV might contribute to tumour vascularization by increased VEGF/VEGFR2 signalling to support the energy requirements of transiently infected cells and to promote tumour growth during malignant transformation.[64,65] Autocrine VEGF-signalling is an essential component in tumour initiation, contributes decisively to the nutritious supply of normal tissue and tumours and is mediated by the receptor tyrosine kinase VEGFR2 in HNSCC.[11,14,52] However, it remains to be elucidated whether HPV exerts specific effects on VEGFR2 expression and further on the regulation of vascularization in OPSCC.

The present study aimed to identify specific VEGFR2 expression patterns in HPV-positive and HPV-negative OPSCC and showed that the expression of VEGFR2 was not only limited to blood vessels but was also present in tumour cells, including cancer stem cells, with HPV-status dependent differences in VEGFR2 expression. This observation shows that VEGFR2 signalling plays a critical role in the progression of OPSCC. *"HPV-positive OPSCC with particularly high VEGFR2 expression were associated with poor prognosis, supporting the prognostic significance of deregulated VEGF signalling for OPSCC patients.*

*Since oxidative stress (OS) is known to induce VEGF expression and it was previously shown that both HPV-positive and -negative OPSCC present with subgroups overexpressing OS signatures going along with poor prognosis, NRF2 expression and analysis of its target gene AKR1C3 serving as a read-out for activated OS signatures was included in this study.[66,67]"* [44]

## 4.1 VEGFR2 expression in blood vessels of HPV-positive and -negative OPSCC

Previous studies which analysed the expression of VEGFR2 in blood vessels of HNSCC, so far, were not focused specifically on OPSCC and no data about HPV status are available.[13,51] The present study focused on primary OPSCC with known HPV status. *"The expression patterns of VEGFR2 in blood vessels of tumour regions and tumour-free regions both in HPV-positive and HPV-negative OPSCC were analysed by densitometric analysis.*

*The results strongly imply that VEGFR2 may be upregulated in endothelial cells of blood vessels supplying nutrients to HPV-positive tumour regions, and that this is not the case in HPV-negative OPSCC. Therefore, our observation might suggest that angiogenesis may be upregulated under the influence of HPV by regulating the expression of VEGFR2 in blood vessels. However, further studies have to clarify by which mechanisms HPV-positive tumour cells might regulate increased VEGFR2 expression in surrounding tumour blood vessels.*

*This hypothesis is furthermore supported by the observation that HPV-positive OPSCC tend to have a higher density of VEGFR2-expressing blood vessels compared to HPV-negative tumours (Fig. 2). While this analysis did not reach significance, the absolute number of tumours*

*with a high density of VEGFR2-expressing blood vessels, however, proved to be significantly higher (Table 1). This key observation is supported by xenograft models in which HPV-positive and -negative cells were incubated under hypoxic conditions and then applied to nude mice.[68] Those mice that received HPV-positive tumour cells showed a higher density of neo-blood vessels, which resulted in improved blood supply and thus less hypoxic tumour areas, which was paralleled by lower mRNA expression of hypoxia-responsive genes such as HIF-1α, GLUT-1 and VEGF-A. Moreover, in that study tumour samples were analysed by immunohistochemical detection of neo-blood vessels using the vascular endothelial cell proliferation marker CD105. This led to the observation of HPV-positive OPSCC having higher numbers of blood vessels."* [44]

Therefore, in the present study it was considered analysing the activation status of VEGFR2 to detect only angiogenically active blood vessels by immunohistochemical staining with antibodies directed against phosphorylated VEGFR2 at Tyr 951, as this modification plays a significant role in tumour angiogenesis and tumour growth.[46] However, no specific immunoreactivity could be detected using the only available antibody suitable for immunohistochemistry thus far, (monoclonal Phospho-VEGFR2 (Tyr951) (15D2); #4991 Cell Signaling; data not shown).

*"Expression of CD31 in endothelial cells modulates cell adhesion, endothelial cell migration and angiogenesis.[66] However, CD31 only gives a static representation of vessel density, whereas VEGFR2 expression may better reflect the physiological stimulus for endothelial growth.[67] In HPV-negative tumours, VEGFR2 and CD31 only partially colocalised in the endothelium of blood vessels and capillaries. The strong colocalisation of CD31 and VEGFR2 in blood vessels and capillaries of HPV-positive OPSCC, however, indicates that HPV induces a highly upregulated angiogenic activity, pointing to significant differences in angiogenesis based on HPV-status.[50,69] This upregulated angiogenic activity may result in an improved response to radiochemotherapy, as individual tumour cells may be more accessible by the bloodstream, leading to a more favourable prognosis compared to HPV-negative OPSCC.[5,70] Mechanistically, increased perfusion delivers oxygen that promotes ROS / free radicals essential for the induction of radiation-induced DNA damage upon radiotherapy thus making cells more accessible for the influx of chemotherapeutic agents.*

*On the other hand, an improved radiation response may also be achieved by blocking VEGFR2 through anti-VEGF therapy.[71,72] Typically, epithelial tumours can respond to radiotherapy with growth factor-driven revascularization, including increased VEGFR2 expression, which may be prevented by anti-VEGF therapy that inhibits revascularization. This, in turn, would lead to increased blood flow and thus a better oxygen supply, resulting in increased ROS formation during radiation therapy and increased flooding of the chemotherapy to the already existing tumour cells. Due to the lack of new blood vessel formation, on the other hand, the formation*

*of new tumour tissue is prevented.[70-72] In this study, upregulation of VEGFR2 and the OS marker NRF2 and AKR1C3 were associated with unfavourable prognosis in HPV-positive OPSCC."* [44]

## 4.2 VEGFR2 expression in tumour cells of HPV-positive and -negative OPSCC

*"In addition, this study could show that VEGFR2 is also expressed in tumour cells. Both HPV-positive and -negative OPSCC presented with a heterogeneous expression pattern including low, moderate, and high VEGFR2-immunoreactivity. This may suggest that aberrant VEGFR2 expression in tumour cells, together with its downstream signalling pathways, may be involved in other besides angiogenesis. However, mutations in VEGFR2 do not seem to be of relevance, as they only show low alteration rates in HNSCC (2.3 % in the TCGA cohort, cBioPortal, data not shown).[73,74] Therefore, other mechanisms like varying differentiation states of tumour cells within one tumour or areas which have a need for improved nutrients and oxygen supply than other regions might correlate with increased VEGFR2 expression levels.*

*Densitometric analysis showed a significantly higher expression of VEGFR2 in HPV-negative tumour cells. This may suggest that the differential expression of VEGFR2 between HPV-positive and -negative tumour cells exhibits crucial cell biological differences.*

*Several oncogenes such as the epidermal growth factor receptor (EGFR) and oncogenic transcription factors such as c-myc are capable to upregulate VEGF expression. Furthermore, wild-type TP53 indirectly represses VEGF.[75] However, HPV16-E6 seems to induce VEGF expression independently of TP53 inactivation, using the SP1 transcription factor for E6-mediated induction of the VEGF promoter.[15] This could also be supported by our observation that VEGFR2 was frequently translocated into the nuclei of HPV-positive but less frequently into the nuclei of HPV-negative tumour cells. It is known that VEGFR2 is translocated to the nucleus of neoplastic cells upon phosphorylation and that VEGFR2 therein may interact with transcription factors such as SP1 to regulate gene transcription.[76,77] Biologically, this might be a self-enhancing mechanism in response to hypoxia and/or OS. In vitro studies using HeLa cells showed that the fraction of nuclear-positive cells increased due to hypoxic stimulation.[76] Degradation of VEGFR2 is furthermore mediated by recruitment of the E3 ubiquitin ligase β-Trcp1 followed by polyubiquitination and delivery to the proteasome.[78] β-Trcp1 is upregulated by HPV16-E7 expression in vitro.[79] Moreover, β-Trcp1 is also involved in the regulation of HIF1α, the WNT/β-Catenin and the PI3K/AKT pathway.[80,81] β-Trcp1 also provides an alternative way to regulate the NRF2/OS pathway for proteasomal degradation via Keap1/Cul3.[82] Therefore, additional side effects may exist, especially considering that all these signalling pathways often present with alterations especially in HPV-negative OPSCC.*

*Noteworthy, a subpopulation of tumour cells presented with high VEGFR2 expression levels. Based on their localization in a perivascular niche adjacent to endothelial cells, it may be suggested that these cells are CSCs. It is known that the amount of this stem cell population and their self-renewal can be regulated by autocrine VEGF/VEGFR2 signalling.[52,83] Double immunostaining with the tumour stem cell marker ALDH1A1 maintaining CSC properties indeed showed colocalisation of VEGFR2 with ALDH1A1. This suggests that these cells might be CSCs localized in the proposed perivascular niche.[84]*

*In line with studies analysing CSCs in comparison of HPV-positive and -negative OPSCC, this study showed a higher number of ALDH1A1$^+$/VEGFR2$^+$ in HPV-positive OPSCC.[85,86] It was recently shown that HPV16 is capable of modifying the phenotype of infected CSCs by increasing the pool of migratory CSCs through expression of HPV16-E6E7 in vitro and in HPV-positive OPSCC.[30] Furthermore, autocrine VEGF/VEGFR2 signalling enhances tumour invasion and survival by promoting processes crucial for CSCs like dedifferentiation and an epithelial-mesenchymal transition phenotype.[52] Furthermore, VEGFR2 can regulate epithelial tumour stem cell migration.[87] Taken together, the higher number of ALDH1A1 and VEGFR2 colocalizing tumour cells in HPV-positive OPSCC might indicate that these tumours are associated with a higher number of migratory CSCs."* [44]

## 4.3 Correlations of the clinicopathological parameters

In the present collective, with an average age of 60 years, most of the HPV-positive cases were male, which corresponds to the data given in the current literature where this is often argued to be because men are more likely to have an oral HPV infection than women.[1,6,21,28]

Data of epidemiological assessments show that smaller T-stage is associated with HPV-positive disease in HNSCC and better OS.[28,29] In the present collective, high T-stage correlated with worse OS in all cases and pT1/pT2 tumours accounted for 56% of all HPV-positive tumours. Possibly due to low collective size, T-stage did not reach significance in separate correlations of both HPV-positive and -negative groups in this study.

In line with data in the literature, the HPV-positive patients in the present collective were less likely to use alcohol (31.4% never drink) than HPV-negative patients (0% never drink).[19,21] However, no significance was reached when comparing low-risk (until 1 g/d) and high-risk drinking profiles (more than 1 g/d up to abuse), since most HPV-negative patients (66.7%) in the analysed collective were no heavy drinkers and therefore classified as low risk.

The risk factor nicotine consumption is also less prevalent in HPV-positive patients, so there was a significant correlation between the parameter nicotine and HPV status.[1,19] Data on the average non-smoking rate of 34.4% in the HPV-positive group and 4.8% in the HPV-negative group are consistent with those in the literature.[88]

Furthermore, HPV-status was associated with a higher number of tumours with a high density of VEGFR2-expressing blood vessels and low VEGFR2 protein expression of tumour cells.

The unfavourable prognosis of HPV-positive tumours that exhibit high VEGFR2 expression and OS-marker emphasizes the clinical relevance of the biological differences of the OPSCC entities (see section 4.1).

## 4.4 Clinical relevance of the results

To improve patient's prognosis, the characterisation of potential molecular targets between HPV-positive and HPV-negative OPSCC is substantial to develop personalized therapeutic approaches. Therefore, the present study investigated the occurrence and distribution of the angiogenic receptor VEGFR2 concerning differences between the two different OPSCC types. Anti-angiogenic agents are currently beneficial in the treatment of several solid tumours but remain experimental in HNSCC.[70] Clinical trials in metastatic/relapsed HNSCC evaluating monotherapy of tyrosine-kinase inhibitors (TKIs) such as sunitinib and sorafenib, which both include VEGFR2 as target, presented with low success, whereas combinations of TKIs with cetuximab or chemotherapy showed some promising results.[70,89]

At present, the combination therapy of anti-angiogenic therapeutics with immune checkpoint inhibitors is of great interest. Clinical phase 1 trials combining ramucirumab (first of its class monoclonal antibody against VEGFR2) with pembrolizumab (PD-1 inhibitor) and lenvatinib (TKI) with pembrolizumab showed promising preliminary efficacy.[89] Ongoing clinical phase 2 trials test the combination therapy of ramucirumab plus pembrolizumab (NCT03650764) and bevacizumab (a monoclonal antibody against VEGF) with atezolizumab (a monoclonal antibody against PD-1 ligand 1; NCT03818061).

One in vitro study tested the HPV-status dependent effects of sunitinib and sorafenib on the VEGFR2 expression in in two HNSCC cell lines (p16-negative) and one (p16-positive) cervical cell line. Interestingly, they observed that the HPV-positive cells had a higher sensitivity to these small molecule drugs.[90] The fact that HPV upregulates angiogenic factors and VEGFR2 downstream pathways have an essential role in endothelial and tumour cell proliferation, invasion, apoptosis regulation and CSC functions also shows possibilities to intervene in this area.[14,52,69] An interesting therapeutic approach by Gao et al. tested the use of a DNA vaccine which contains HPV16 E6E7 and VEGFR2 fusion gene in a mouse model and resulted in a promising synergistic antitumor and anti-angiogenetic effect.[91]

## 4.5 Strengths and limitations of the study

The present study has several strengths. One is that the object studied, VEGFR2, is a potential target for the treatment of OPSCC. From this, a second strength of the study can be derived, as the research data stratified with the clinical data have additional clinical prognostic and diagnostic value. Further, it is a strength that the available clinical data came from long-term patient follow-ups. Finally, a major strength is the direct comparison of the molecular

expression profile between blood and cancer cells from the immunohistochemical analysis of the OPSCC samples.

The results of this thesis also have some limitations. First, 56 patients is a relatively small sample size, however, sufficient sample size was determined before analysis with a power of 90% and a significance level for beta-error of 0.05. Nevertheless, the interpretation of the colocalisation analysis may be limited because double immunofluorescence stainings of VEGFR2 with tumour cell marker ($p16^{INK4A}$ and p53 respectively), CD31 or ALDH1A1 were performed on sub-series with limited case numbers.

Second, the analysis of blood vessel density could not be performed with antibodies directed against phosphorylated VEGFR2 and therefore the interpretation on angiogenic activity may be limited (see section 5.1).

Third, the results cannot describe mechanisms that underly the associations between HPV-status and VEGFR2 expression in OPSCC, which were determined for the first time in this study. Consequently, many hypotheses in the discussion section are mainly indicated as a suggestive. However, the study forms the basis for future studies to investigate the molecular mechanisms.

## 4.6 Conclusion and suggestions for further work

*"In conclusion, the present study identified a distinct molecular protein expression profile of VEGFR2 in HPV-positive and HPV-negative OPSCC. In the HPV-positive group, significant differences in VEGFR2 expression levels between blood vessels of tumour regions compared to tumour-free regions were observed. In contrast, HPV-negative OPSCC presented with significantly higher VEGFR2 expression levels in tumour cells. Based on this observation, two different HPV-status dependent phenotypes of VEGFR2 signalling may exist, possibly triggered by hypoxia and/or oxidative stress (Fig. 5). Future studies should focus on unravelling the molecular basis of mechanisms involved in the differential regulation of VEGFR2 expression between HPV-positive and -negative OPSCC in tumour supporting blood vessels and tumour cells. Such studies could prove to be of pivotal importance for patient outcome when anti-VEGF therapies in the treatment of OPSCC are considered.*

*Furthermore, the data indicate that VEGFR2 may play a regulatory role in CSCs of HPV-positive OPSCC. CSCs are thought to be responsible for treatment failure in anti-cancer therapy. Experimental validation of the regulatory role of VEGFR2 in stem cell migration and dedifferentiation, especially in CSCs of HPV-positive OPSCC, will further contribute to the understanding of unfavourable development of (distant) metastases and recurrence."* [44]

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# 6. Pre-publications of results

## 6.1 Congress contribution

Uzun, S.; Korkmaz, Y.; Würdemann, N.; Arolt, C; Puladi, B; Siefer, OG; Klußmann, JP; Hübbers, CU. HPV status and its correlation to the expression of VEGFR2 in cancer cells and in blood vessels of OPSCC. *Laryngo-Rhino-Otologie* 2019; 98(S02): 85 - 85. doi:10.1055/s-0039-1686087 (Lecture at the 90th Annual Meeting of the DGHNO-KHC 2019, Berlin)

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**Abstracts / Oncology**

Uzun, S; Korkmaz, Y; Würdemann, N; Arolt, C; Puladi, B; Siefer, OG; Klußmann, JP; Hübbers, CU

# HPV-status and its correlation to the expression of VEGFR2 in cancer cells and in blood vessels of OPSCC

Abstract- und Posterband – 90. Jahresversammlung der Deutschen Gesellschaft für HNO-Heilkunde, Kopf- und Hals-Chirurgie e.V., Bonn – Digitalisierung in der HNO-Heilkunde

29. Mai – 01. Juni 2019, Estrel Congress Center Berlin

Präsident: Prof. Dr. med. Stefan Dazert, Bochum

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**Introduction:**

Up to 50% of oropharyngeal squamous cell carcinoma (OPSCC) is associated with Human Papillomavirus (HPV) type 16 and it is known that HPV-positive and HPV-negative OPSCC exhibit different mutation patterns and expression signatures. Vascular endothelial growth factor receptor 2 (VEGFR2) regulates tumor angiogenesis. However, the effect of HPV-Infection on VEGFR2 expression in correlation to tumor angiogenesis in OPSCC is unknown.

**Methods:**

Paraffin sections of OPSCC samples (n = 46) with known HPV-status were incubated with VEGFR2 immunohistochemically. The colocalization of VEGFR2 with p16 and p53 in tumor cells and with CD31 in blood vessels was analyzed with double immunostaining. The number of VEGFR2-stained blood vessels and staining intensity of VEGFR2 in tumor cells were quantified by QuPath bioimage software. Results were correlated with clinicopathological data.

**Results:**

VEGFR2 expression was detected in numerous blood vessels of tumor regions as well as in tumor cells. Our statistical analysis showed significant differences in staining intensities between HPV-positive and -negative tumor cells ($p = 0.0103$). HPV-infection induces a significant downregulation of VEGFR2 in cancer cells compared to HPV-negative OPSCC. No significant differences in the number of VEGFR2-positive capillaries between HPV-negative and HPV-positive OPSCC were observed.

**Conclusion:**

We conclude that an increase of VEGFR2 expression in tumor cells is correlated to HPV-status. However, in the capillaries of OPSCC, vessel density is not affected by HPV infection. The high number of VEGFR2-positive capillaries in OPSCC is not a direct consequence of altered VEGFR2 expression in tumor cells. In the tumor milieu, the effects of HPV in OPSCC are regulated in a cell-specific manner.

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## 6.2 Publication

Uzun, S.; Korkmaz, Y.; Wuerdemann, N.; Arolt, C.; Puladi, B.; Siefer, O.G.; Dönmez, H.G.; Hufbauer, M.; Akgül, B.; Klussmann, J.P.; Huebbers, C.U. Comprehensive Analysis of VEGFR2 Expression in HPV-Positive and -Negative OPSCC Reveals Differing VEGFR2 Expression Patterns. *Cancers* 2021,*13*,5221. https://doi.org/ 10.3390/cancers13205221