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CXCR4-CXCL12 axis in head and neck squamous cell carcinoma regarding HPV status

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Abbreviation

AJCC	American joint committee on cancer
Aqua dest.	Distilled water
cDNA	Complementary deoxyribonucleic acid
CXCR	C-X-C chemokine receptor
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
ECM	Extracellular matrix
EMT	Endothelial-to-mesenchymal transition
ENE	Extranodal extension
FBS	Fetal bovine serum
FDA	Food and drug administration
FFPE	Formalin-fixed paraffin-embedded
FNA	Fine needle aspiration
GDP	Guanosine diphosphate
GTP	Guanine nucleotide triphosphate
GPCR	G protein-coupled receptor
HIV	Human immunodeficiency virus
HNSCC	Head and neck squamous cell carcinoma
HRP	Horseradish peroxidase
HPV	Human papillomavirus
IARC	International agency for research on cancer
IC	Induction chemotherapy
IHC	Immunohistochemistry
IKK	IκB kinase
LRC	Loco-regional control
M	Mol
MAPK	Mitogen-activated protein kinases,
MEK	MAPK kinase
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
N-terminal	Amino-terminal
NF-κB	Nuclear factor-κB
OPSCC	Oropharyngeal squamous cell carcinoma
OSCC	Oral squamous cell carcinoma
PAK	P21-activated protein kinases
PBS	Phosphate-buffered saline
PBSF	Pre-B-cell-growth-stimulating factor
PCR	Polymerase chain reaction
PET	Fludeoxyglucose-positron emission tomography
PI3K	Phosphoinositide-3 kinase
PK	Protein kinase
pRB	Retinoblastoma protein

RCA	Renal cell carcinoma
rcf	Relative centrifugal force
RNase	Ribonuclease
rpm	Revolutions per minute
RRID	Research resource identifiers
RT	Radiotherapy
RT-CHX	Radio-chemotherapy
SD	Standard deviation
SDF	Stromal derived factor
SDS	Natriumdodecylsulfat
SEER	Surveillance epidemiology and end results
SEM	Standard error of the mean
TIMP	Tissue inhibitors of metalloproteinase
uPA	Urokinase-type plasminogen activator
UPS	Ubiquitin–proteasome system
WHO	World health organisation
µ	Micro (10^{-6})

1 Introduction

1.1 Head and neck squamous cell carcinoma

1.1.1 Definition and epidemiology

Head and neck squamous cell carcinomas (HNSCCs) are epithelial-derived tumors of mucosa linings of the upper aero-digestive tract including the oral cavity, the oropharynx, the nasal cavity, paranasal sinuses, the nasopharynx, the hypopharynx and the larynx (Taneja et al. 2002). HNSCCs are classified as the seventh most common cancer worldwide, with annually approximate 800,000 new diagnosed cases (Bray et al. 2018). HNSCCs are a highly heterogeneous disease with a significant discrepancy of incidence in distribution of geographical region (Hussein et al. 2017). The age adjusted incidence for males decreased significantly between 1975 and 2002 in comparison with females from the National Cancer Institute's Surveillance, Epidemiology and End Results Program (SEER) (Yerger, Przewoznik, and Malone 2007). The incidence of HNSCC is lower among Blacks than Whites for the first time in the United States since around 2008 (Fakhry et al. 2018).

1.1.2 Etiology

Tobacco and/or alcohol abuse, infection with high-risk human papillomaviruses (HPV) and genetic aberration for HNSCC are considered being the main risk factors of HNSCC (Gillison et al. 2015). Although the individual contribution of each risk factor was hard to evaluate, alcohol consuming was first identified as a risk factor for cancers of the oral cavity, oropharynx, hypopharynx, and larynx from The International Agency for Research on Cancer (IARC)(Cancer 1988). A recent study with almost 4,000 patients of newly diagnosed OPSCC demonstrated a relatively increased incidence in OPSCC without tobacco and alcohol exposure (Dahlstrom et al. 2013). Furthermore, HPV-related HNSCC is shown an increased incidence of 25% in the U.S. in last decades, predominantly occurring among young individual(<45) (Carvalho et al. 2005). Due to the distinction of HPV-associated HNSCC with unique etiology, molecular pathogenesis, clinical manifestation and therapeutic sensitiveness, the latest TNM classification firstly differentiated between HPV-associated and non-HPV-associated OPSCC clinically and pathologically (Hoffmann and Tribius 2018). Other epigenetic less known risk

factors such as chronic sun exposure, inflammation, and/ or metabolic disturbances, family history of HNSCC, and older age are associated to the development of HNSCC (Warnakulasuriya 2009).

1.1.3 Diagnosis and HPV-associated classification

In initial diagnosis, physical examination, fine needle aspiration (FNA) cytology and imaging studies including computed tomography (CT) scan, magnetic resonance imaging (MRI), [18F] Fludeoxyglucose-positron emission tomography (PET) and integrated PET/CT are used to determine the initial extent of disease and stage of HNSCC (De Bree et al. 2012). FNA biopsy is applied to the suspicious lymph node relating primary tumor to provide more accurate information basing on clinical and imaging assessment. In the screening of distant metastases, chest CT and whole-body- FDG-PET are the most utilized imaging diagnostic approaches (Senft et al. 2008). In the follow-up, distant metastases were often missed due to the deficiency of susceptibility in screening technique in these patients reaching loco-regional control.

In the 8th edition of the American Joint Committee on Cancer (AJCC) Staging Manual for clinical staging criteria of HPV-OPSCC, separated clinical and pathological staging was proposed for HPV-OPSCC from head neck cancer and it provided a unique classification for nodal disease (N category) to optimize management strategy and prognosis evaluation in HPV-associated disease (see Appendix).

1.1.4 Treatment and prognosis

According to the latest US National Comprehensive Cancer Network (NCCN) guidelines versions 1.2017, multidisciplinary treatments of sugery, radiation, and chemotherapy alone or with a combination are employed to the management of HNSCC on basis of TNM stage and primary site. Although HPV-associated OPSCC was staged differently compared to HPV-negative tumors from 8th AJCC manual, the today's treatment strategies remain unchanged. Surgery or radiation alone is usually chosen for the treatment in stage I and II HNSCC with curative intent. After surgical resection, adjuvant postoperative radiotherapy (RT) to the local and regional sites of disease is the principal treatments for patients with stage

III or IV HNSCC. In the cases of locoregionally advanced, technically unresectable HNSCC, platinum-based adjuvant radio-chemotherapy (RT-CHX), with or without induction chemotherapy (IC) are preferentially chosen. With the advantages in the chemoreduction before radiotherapy, principally IC is applied to decrease the toxicity of radiotherapy and tumor burden (Gujral et al. 2014). The treatments in the case of local or regional recurrence or distant metastasis with palliative intend ranged from surgery, systemic chemotherapy, single agent chemotherapy, palliative RT and/or supportive care depending on the site of recurrence, tumor burden, and previous therapy (Fulcher et al. 2018). In 2019 the immunotherapy agent pembrolizumab was approved by the US Food and Drug Administration (FDA) as first-line treatment for the patients regarding recurrent or metastatic HNSCC in addition to platinum and fluorouracil. Also, it was reserved for the HNSCC patients with tumors expressing programmed death-ligand 1 (PD-L1) with a combined positive score ≥1 as a single therapeutic agent (Cohen et al. 2019).

Usually, at the time of diagnosis, approximately half of all patients with HNSCC presented with stage III or IVA-B disease accompanied by an estimated 5-year overall survival rate ranged from 10% to 40% (Leibel et al. 1991; Li et al. 2009; Coca-Pelaz, Rodrigo, and Suárez 2012; Bossi et al. 2019). The median survival time in HNSCC patients with distant metastases at diagnosis was reported from 1 to 12 months (Ferlito et al. 2001). Approximate 45% of HNSCC patients with primary diagnoses of distant metastases suffered from loco-regional recurrence in follow-up (Duprez et al. 2017). From SEER database, distant metastasis incidence was reported as 2.82% at presentation (Kuperman et al. 2011). Moreover, distant metastasis plays a decisive role in modifying the therapeutic regimen and prognostic evaluation in HNSCC. Among the clinical prognostic parameters including tumor size, histological grade, nodal status, etc, the locoregional control with nodal status are identified as main risk factors for distant metastasis in HNSCC(Takes et al. 2012).

1.2 HPV-associated HNSCC

1.2.1 HPV introduction

Human papillomaviruses (HPVs) are a family of non-enveloped double-stranded DNA viruses that specifically target the basal cells of cutaneous and mucosal epithelia and establish a persistent infection (Zur Hausen and De Villiers 1994). HPVs productively replicate in the

infected epithelium causing chronic lesions, which can lead to carcinogenesis relating to viral subtypes (Münger and Howley 2002). So far, there are over 200 subtypes of the HPVs. According to the ability of HPV to infect epithelial cells, then they are classified into high-risk types and low-risk types (Yeo-Teh, Ito, and Jha 2018). HPV infection is prominently attributed to types of HPV-16 and HPV-18 (Mirabello et al. 2018).

The HPV genome consist of the early gene-coding region, late gene-coding region, and the non-coding control region, which is involved in the regulation of viral replication, transcription, and oncogenesis (Egawa et al. 2015). The induction of HPV early protein E6 and E7 coded by the high-risk HPVs kidnaps the cellular ubiquitin–proteasome system (UPS) to increase proliferative potential and evasion of apoptosis (Forcier and Musacchio 2010). The HPV E7 protein prominently binds and degrades the tumor suppressor protein, Retinoblastoma protein (pRb), leading to an overexpression of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ in the HPV-infected tissue (Boyer, Wazer, and Band 1996). Clinically, HPV-DNA testing and $p16^{Ink4a}$ immunohistochemical staining are applicable in identifying HPV positive tumors. In modified 8th AJCC/UICC, $p16^{Ink4a}$ was recommended as HPV surrogate marker with the cutoff point more than 75% histological overexpression and not lower than 2/3 staining intensity (Lydiatt et al. 2017).

1.2.2 HPV in oncogenesis

Persistent infection status with high-risk types of HPV is considered as a progression in the transformation of high-grade lesions or cancer (Yeo-Teh, Ito, and Jha 2018). The correlation between HPV infection and carcinogenesis gained considerable focuses as the prevalence of HPV was confirmed in a variety of carcinomas (Brianti, De Flammineis, and Mercuri 2017). The high-risk HPV E6 and E7 oncogenes are considered the crucial factor in primary human keratinocyte immortalization and initiation and subsequent progression of carcinogenesis. The oncoprotein E6 is known to degrade tumor protein p53, thereby, block p53 downstream pathways to interfere cell cycle arrest in G1 phase and inhibit cell apoptosis (Wiest et al. 2002). Moreover, the HPV E7 oncoprotein degrades the pRb tumor suppressor protein, forcing cell into S-phase to provide a growth advantage and promote malignant transformation (Wiest et al. 2002). Oncogenic HPV types showed an affinity for infecting the immature squamous cells (Handler et al. 2015). Among the types of HPVs, HPV 6, 11, 16, and 18 are known by the functions in infecting mucosal epithelial cells of the oral cavity, oropharynx, and

uterine cervix (Schiffman, Clifford, and Buonaguro 2009). Clinically, HPV positive tumors usually present with a small T stage but with an advanced nodal stage (Hafkamp et al. 2008; de Martel et al. 2017).

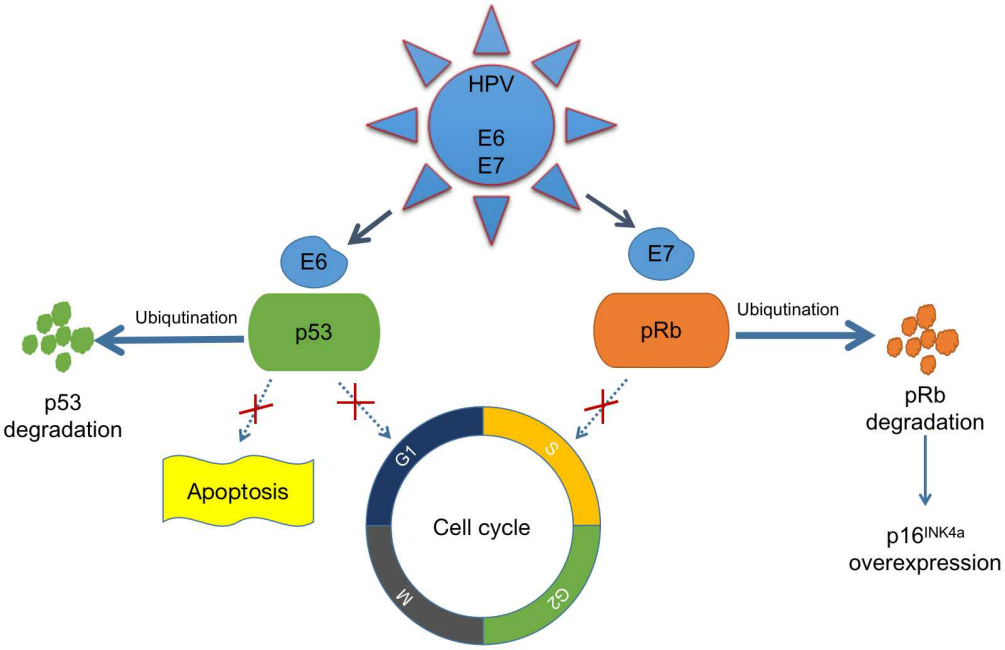

Figure 1.1 The role of human papillomavirus (HPV) in the carcinogenesis

HPV E6, E7 protein respectively binds p53 tumor suppressor protein and pRb cell cycle regulatory protein through the degradation of p53 and Rb to interfere cell cycle arrest and apoptosis (Wiest et al. 2002).

1.2.3 HPV in HNSCC

Despite reported discrepancy of prevalence between European, American and Asian continent, the HPV-associated HNSCC keep rising significantly, while the non-HPV-associated HNSCC had declined over 30 years (Boscolo-Rizzo et al. 2018; Shaikh, McMillan, and Johnson 2015). The data from the SEER revealed that the prevalence of non-HPV-associated cancers decreased by 50% from 1988 to 2004, whereas HPV-associated OPSCC increased by 225% in the United States (Pytynia, Dahlstrom, and Sturgis 2014). This data underlined the universality of HPV infection as the primary cause of OPSCC. From collected evidence of HNSCC, HPV subtype 16 accounts for around 90% of HPV-associated HNSCC (Ndiaye et al. 2014). HPV-associated HNSCC manifested a distinction regarding anatomic sites, in which oropharyngeal SCC accounted for 33.6 % in comparison to the larynx (20.2%) and the oral cavity (22.2 %) (de Martel et al. 2017). Moreover, a correlation was confirmed

between the positivity of HPV-16 in HNSCC patients and the local metastatic lymph nodes, and an advanced tumor stage at primary diagnosis (Goldenberg et al. 2008). HPV-16 infection was considered as an independent risk factor for HNSCC, therefore the HPV status is used to be a prognostic marker in epidemiologic investigation (Gillison et al. 2015). Several studies have disclosed that HPV-associated HNSCC patients had a significantly improved overall and disease-free survival in comparison to non-HPV-associated HNSCC patients (Fakhry et al. 2008; Ang, Harris, Wheeler, Weber, Rosenthal, Nguyen-Tân, et al. 2010; Marur et al. 2010; Ahmadi et al. 2019; Bychkov et al. 2016). The more favorable outcome in HPV-associated OPSCC patients was associated with improved therapeutic response to induction chemotherapy and chemo-radiation in comparison to non-HPV-associated patients (Fakhry et al. 2008). Although loco-regional control was also significantly favorable in HPV-associated OPSCC, the incidence of distant metastasis in HPV-associated OPSCC after 2 years was significantly higher than in the non-HPV-associated OPSCC patients (Benson et al. 2014). A meta-analysis pointed out a significantly improved 5-year overall survival (OS) in HNSCC patients with HPV (+) / p16 (+) status in comparison to non-HPV/p16 associated subgroups (Coordes et al. 2016). More research focusing on viral integration events, genomic alteration, the biological properties is required to further explain the correlation with clinical implications to optimize the therapeutic intervention in future.

1.3 CXCR4-CXCL12 axis in human cancers

1.3.1 Chemokines and their receptors

Chemokines, as superfamily of small molecular proteins, are commonly classified as CXC, CC, C, and CX3C according to their conserved N-terminal cysteine residues. Chemokine receptors are divided into G protein-coupled receptors (GPCRs) and the atypical chemokine receptors (ACKRs). Functionally, GPCRs can activate distinct signalling pathways through other G-protein subtypes, e.g. the Gα, Gβ, and Gγ subunits, leading to different cellular responses after ligands bind to GPCRs (Rossi and Zlotnik 2000; Bachelerie et al. 2014). Among these chemokine receptors, increased focus on CXCR4 was based on its established pivotal role in physiological and pathological process.

1.3.2 CXCR4 and its ligand: CXCL12

CXCR4 has an extracellular N-terminus, 7-transmembrane G-protein coupled receptor by three extracellular and three intracellular loops. CXCR4 is ubiquitously expressed in the peripheral blood, bone marrow, lymph nodes, liver, lung, brain, and other organs (Kim et al. 2007). CXCR4 takes part in physiological processes such as immunity, haematopoiesis, neurogenesis, angiogenesis as well as in pathological processes including human immunodeficiency viruses (HIV) infection, autoimmune diseases and tumor diseases (Domanska et al. 2013).

The CXC chemokine ligand-12, also known as pre-B-cell-growth-stimulating factor (PBSF) or stromal derived factor 1 (SDF-1) can selectively be bound by CXCR4 (Shirozu et al. 1995). CXCL12 is diffusely expressed in adult tissues such as the lung, brain, liver, colon, skeletal muscle, heart, kidney, and bone marrow (Cinamon, Shinder, and Alon 2001). CXCL12 is disclosed to perform in physiological processes including embryogenesis, haematopoiesis, organogenesis, vascularization, and tissue regeneration (Janssens, Struyf, and Proost 2018). In adult organisms, CXCL12 fulfilled its functions in the different physio-pathological processes such as hematopoietic cell trafficking, adhesion, immune surveillance, and conceivably regulating the initiation and spreading of cancer metastasis (Janssens, Struyf, and Proost 2018; Lataillade, Domenech, and Le Bousse-Kerdilès 2004).

CXCL12 binds to CXCR4 through the heterotrimeric G protein activation by the exchange of guanosine diphosphate (GDP) for guanine nucleotide triphosphate (GTP) and the dissociation into the α and the βγ subunits with a course of desensitization (Oldham and Hamm 2008). Multiple cellular signal transduction pathways were demonstrated via CXCR4-CXCL12 activation to regulate cell survival, proliferation, migration and chemotaxis (Pozzobon et al. 2016; Teicher and Fricker 2010b). Following the binding of CXCR4 and CXCL12, activated by both Gβγ and Gα subunits, activation of phosphoinositide-3 kinase (PI3K) triggered the activation of the protein kinase B (PKB or Akt), p21-activated protein kinases (PAK) pathways in cell polarization and actin polymerization contributing to cell migration and chemotaxis (Teicher and Fricker 2010b; Barbero et al. 2003). Moreover, CXCR4-CXCL12 binding causes CXCR4 desensitization, mediates internalization of the receptor by GPCR kinase (GRK)-dependent phosphorylation and induces activation of down-stream β-arrestin-dependent cell signalling pathway (ERK-1/2), which regulates cell migration (Marchese 2014; Chen et al. 2012). In turn, the mitogen-activated protein kinase (MAPKs) signalling could be activated through the CXCR4-CXCL12-induced activation of MEK

(MAPK kinase)/nuclear factor-κB(NF-κB) signalling pathways, promoting cell migration (Sun et al. 2002). Furthermore, that CXCR4-CXCL12 signalling in HNSCC induces NF-κB signalling pathway activation through PKC/IκB kinase (IKK), PI3K/Akt and ERK/MAPK independent signalling pathway was implicated in regulating the invasion and metastasis of cancer cell (Rehman and Wang 2008; Rehman and Wang 2009). Subsequently, the involvement of CXCR4-CXCL12 axis in epithelial mesenchymal transition (EMT) via PI3K/Akt pathway activation in HNSCC had been established (Yoon et al. 2007; Taki et al. 2008).

1.3.3 CXCR4-CXCL12 axis in HNSCC

Substantial evidence demonstrated that the CXCR4 and its ligand CXCL12 were involved in the oncogenesis on the basis of their overexpression in most epithelial and mesenchymal cancers including ovarian, prostate, breast cancer, melanoma and neuroblastoma et al (Mao, Fan, and Liu 2017; Kim et al. 2008; Hall and Korach 2003; Taichman et al. 2002; Geminder et al. 2001). Furthermore, the role of CXCR4-CXCL12 axis was widely revealed concerning their involvement in cell cycle progression, invasiveness and migration(Albert et al. 2013). In HNSCC primary tumors, CXCR4 was investigated to be highly expressed in HNSCC primary carcinoma than in normal epithelium (Ueda et al. 2010; Albert et al. 2013; Ishikawa et al. 2006b; Katayama et al. 2005). From the studies, the specific expressed CXCR4 positivity rate in primary tumor of HNSCC varied from 30% to 100% in primary tumor of HNSCC (Al-Jokhadar, Al-Mandily, Zaid, and Maalouf 2017; Tan et al. 2008; Ishikawa et al. 2006b; Delilbasi et al. 2004). The expression of CXCR4 was mainly detected in the cell membrane and cytoplasm of the HNSCC cells through IHC staining (Albert et al. 2013; Knopf et al. 2017). Several reports announced the correlation between the clinicopathologic parameters and the expression of CXCR4 in primary tumor, including tumor size, tumor histologic type, TNM classification and locoregional and systematic recurrence (Knopf et al. 2017; Yin and Gao 2007). Moreover, high expression of CXCR4 in primary HNSCC cells as well as in metastatic cancer cells of lymph nodes were detected (Uchida et al. 2007; Al-Jokhadar, Al-Mandily, Zaid, and Maalouf 2017). Subsequently, a correlation was established between CXCR4 expression in primary tumor and nodal status instead of T status (Ishikawa et al. 2006b). CXCR4-positive patients were shown significant worse 5-year survival and higher

risk of local and systemic recurrence in comparison to CXCR4-negative HNSCC patients (Katayama et al. 2005; León et al. 2016; Knopf et al. 2017; Uchida et al. 2007). Moreover, HNSSC patients who suffered metachronous distant metastasis was demonstrated to have a high CXCR4 expression in the primary tumor (Katayama et al. 2005).

CXCL12 was demonstrated to predominantly express in adjacency of cancer cells such as stromal cells and lymphocytes instead of in cancer cells themselves in HNSCC (Clatot et al. 2015; Tan et al. 2008) The two ways of CXCL12 secretion with autocrine by cancer cell itself and paracrine by adjacent stromal cells and lymphocytes strongly suggested the potential of CXCL12 involved in cancer metastasis(Tan et al. 2008). The reported positivity rate of CXCL12 expression in HNSCC primary tumor was from 40% to 93% from the studies (Albert et al. 2013). A recent article demonstrated that HNSCC patients with overexpression of intracellular CXCL12 and CXCR4 of tumor had a higher risk of loco-regional recurrence and a worse overall survival after RT-CHX (De-Colle et al. 2017). Nevertheless, the role of CXCL12 in prediction of prognosis showed inconsistence in HNSCC (Uchida et al. 2007; Zhang et al. 2019; León et al. 2016).

Recently, a finding that expression of CXCL12 in epidermal keratinocytes correlated with HPV-induced cell transformation was confirmed in WHIM syndrome, which associated with the gain-of-function mutated CXCR4 subtype (Balabanian et al. 2005). Moreover, the upregulation of CXCL12 and its receptors CXCR4 and CXCR7 in keratinocytes was regulated by oncogenic HPV16 or HPV18 depending on the viral proteins E6 and E7 (Chow et al. 2010). In particular, in HPV-induced epithelial neoplasia, epidermal neoplastic progression was significantly inhibited by blockage of CXCR4-CXCL12 axis with selective CXCR4 antagonist AMD3100 in transgenic mouse model (Meuris, Gaudin, et al. 2016). Thus, collected evidence indicated strongly the involvement of autocrine signalling of CXCL12 in oncogenic transformation and revealed a therapeutic potential in HPV-induced carcinogenesis.

1.3.4 CXCR4-CXCL12 axis related proteins in HNSCC (TIMP1, TIMP2, MMP2, MMP9 and PLAU)

Matrix metalloproteases (MMPs) are a family of zinc-dependent endopeptidases that regulate physiologic tissue-remodelling processes through the degradation and reconstruction of extracellular matrix (ECM)(Zhang and Chen 2017). Destruction of the ECM through

degradation of its structural components is considered to be a prerequisite for invasion and metastasis relating to enzymatic proteins and MMPs in the initiation of cancer metastasis (Wyckoff et al. 2006; Wolf et al. 2009). Among the MMP family, due to the significantly overexpression of MMP2 and MMP9 in primary tumor and metastatic site from upper aerodigestive tract tumor, increased interests focused on MMP2 and MMP9 in cancer progression (Hong et al. 2000; Dünne et al. 2003). Recently, reported studies demonstrated a correlation between MMPs expression and lymph nodal status as well as prognosis in HNSCC (Heissenberg et al. 1998; Silva et al. 2018; Gontarz et al. 2016). In addition, higher expression of MMP9 levels were disclosed a correlation with T stage and M status in OSCC (Hong et al. 2000). Subsequently, it was demonstrated that the activation of CXCR4-CXCL12 signalling induced the expression of MMP2 and MMP9 in HNSCC cell lines through ERK-1/2 mediated signalling pathway leading to increased adhesion and migration of tumor cells in HNSCC(Samara et al. 2004).

Tissue inhibitors of metalloproteinases (TIMPs) are inhibitors of MMPs activity. Literally, the dynamic balance between MMPs and TIMPs was considered as a key regulatory factor in tumors invasion and metastasis (Werner, Rathcke, and Mandic 2002). Next to their ability to inhibit MMPs, TIMPs also play a role in pathological process. Kurahara et al. presented that primary tumors with metastatic spread showed significantly higher TIMP1 expression than tumors without metastatic spread in OSCC (Kurahara et al. 1999).

The high expression of TIMP1 in primary laryngeal squamous cell carcinoma correlated with poor 5-years prognosis (Ma et al. 2014). Moreover, the decline of MMP9 concentration and changed MMP9/TIMP1 ratio was negatively correlated with lymph node status in postoperative patients of HNSCC (Stanciu et al. 2017). Furthermore, expression of MMP2 and TIMP2 was demonstrated to correlate with local and regional lymph node status in SCC of the oral tongue (Yoshizaki et al. 2001).

The urokinase-type plasminogen activator (uPAs), encoded by the PLAU gene, is known to mediate the conversion of inactive plasminogen into plasmin contributing to wound healing as well as tumor invasion and migration (Pavon et al. 2016; Shi and Stack 2007). In addition, ECM degradation in tumor migration by the release of cellular uPA was proven to be activated by MMP2 and MMP9 (Legrand et al. 2001; Zhan et al. 2020; Kessenbrock, Plaks, and Werb 2010). Moreover, overexpression of uPA/uPAR was demonstrated as an independent prognostic factor in relapse-free survival in HNSCC (Hundsdorfer et al. 2005).

Taken all together, due to their implication in tumor progression and metastatic spread, the interplay of MMPs, TIMPs and PLAU with CXCR4-CXCL12 axis may provide an insight to better understand the potential mechanism underlying the tumor metastasis.

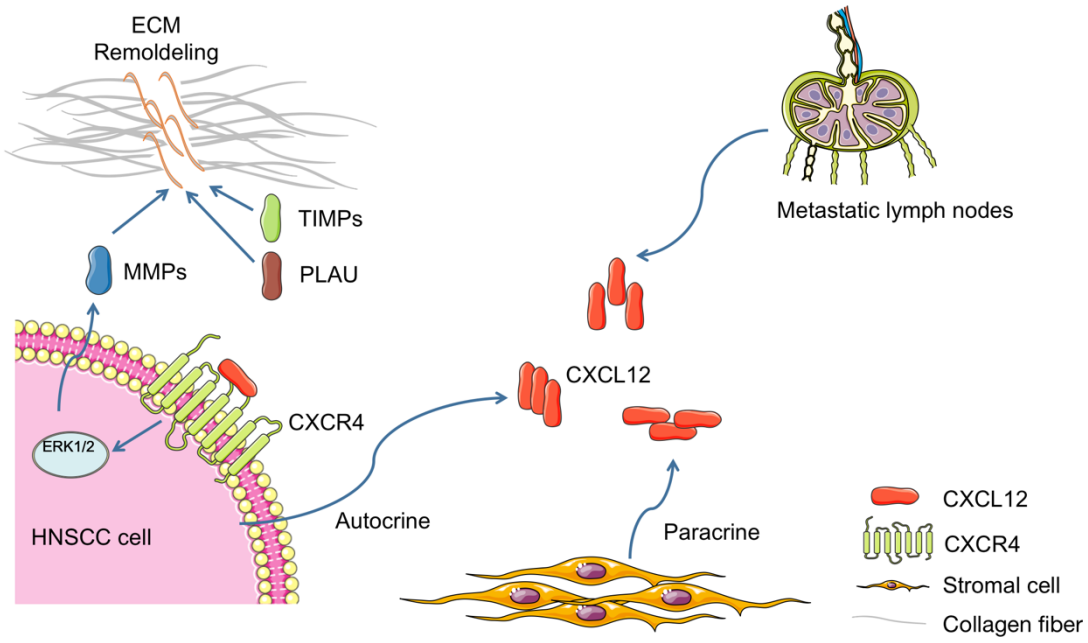

Figure 1.2 The activation of CXCR4-CXCL12 signalling

CXCR4 is expressed in primary HNSCC cells, CXCL12 is not only expressed in primary HNSCC cells in an autocrine way but also in stromal cells and metastatic lymph nodes in a paracrine way. The activation of CXCR4-CXCL12 promotes the ECM remodelling through interplay with PLAU, MMPs and TIMPs (Albert et al. 2013; Knopf, Fritsche, and Li 2017; Kessenbrock, Plaks, and Werb 2010).

2 Aim of study

HPV-associated HNSCC, with a dramatic increased prevalence, sharpened the burden of cancer worldwide. Moreover HPV-associated HNSCC manifested distinct characteristics with more favorable prognosis and better treatment response. Recent revised TNM staging principle referring HPV-associated OPSCC highlighted the importance in understanding the biologic characteristics of HPV in cancer progression to reach better management of tumor. Recently investigations revealed the potential of CXCR4-CXCL12 taking part in the process of carcinogenesis of HPV. Considering the role of CXCR4-CXCL12 performing not only in the progress of HNSCC cell adhesion, proliferation, invasion and migration but also in prognostic value of survival and loco-regional control, proper questions had been rendered as follow:

1. Does the difference of clinical characteristics exist in collected OPSCC cohort regarding HPV status?
2. Does the expression of related proteins of CXCR4-CXCL12 axis differ in OPSCC primary tumors regarding HPV status, accordingly leading to the clinicopathological modification?
3. Is CXCR4-CXCL12 signalling associated with tumor cell proliferation, migration and chemotaxis in HNSCC cell lines regarding HPV status?
4. Do the treatments of cisplatin or/and blockade of CXCR4-CXCL12 signalling take effect on HNSCC cell motility and does the drug-resistance exist in the HNSCC cell lines regarding HPV status?

Thus, the aim of our study is to analyse the clinicopathological features of OPSCC cohort regarding HPV status, further investigate the role of CXCR4-CXCL12 axis in HNSCC cell proliferation, migration and chemotaxis in vitro and establish a potential therapeutic approach in inhibition of HNSCC cell metastasis with regard to HPV status.

3 Material and Methods

3.1 Material

3.1.1 Technical devices

Microscope	Microscope Leica DMI 6000B Camera Leica DFC 425C	Leica Microsystems, Wetzlar, Germany
Centrifuge	5430R Rotor： FA-45-30-11 F-35-6-30 Universal 320R	Eppendorf AG, Hamburg, Germany Hettich GmbH & Co.KG, Tuttlingen, Germany
Weighbridge	Scaltec Recepture SBC21	Scaltec Instruments GmbH, Goettingen, Germany
Spectralphotometer	NanoDrop ND-1000	Thermo Fisher Scientific, Langenselbold, Germany
Thermocycler	CFX96™ C1000 Thermo Cycler	Bio-Rad Laboratories GmbH, Munich, Germany
Power Supply	Power Pac 300 Power Supply	Bio-Rad Laboratories GmbH, Munich, Germany
Water Bath		GFL Labortechnic GmbH, Burgwedel, Germany
Incubator	CO_2-incubator Hera Cell 150i incubator	Thermo Fisher Scientific, Langenselbold, Germany
Benchtop	Hera safe KS	Thermo Fisher Scientific
Multiwell Reader	Multiscan™ FC Microplate	Thermo Fisher Scientific
pH-gauge	pH-Meter 766 Calimatic	Knick Electronic device GmbH & Co.KG, Berlin, Germany
Shaker	MS 2 Mini shaker	IKA Werke GmbH& Co.KG, Staufen, Germany
Heating Blocker	Thermomixer comfort	Eppendorf AG, Hamburg,

		Germany
Microtome	HM 355S automatic Microtome	Thermo Fisher Scientific
Immunohistochemistry Staining System	Ventana BenchMark® GX	Ventana Medical Systems, Tucson, Arizona, USA
Cell Counter	Cellometer Auto T4	Nexcelom Bioscience LLC., Lawrence, USA

Table 3.1 Technical devices

3.1.2 Software

Image Lab Software 6.0	Bio-Rad Laboratories GmbH, Munich, Germany
ND-1000 v3.8.1	Thermo Fisher Scientific, Langenselbold, Germany
CFX Manage Software 3.1	Bio-Rad Laboratories GmbH, Munich, Germany
Cellometer Auto Counter 3.3.9.5	Nexcelom Bioscience LLC., Lawrence, USA
Skanit software 2.5.1	Thermo Fisher Scientific, Langenselbold, Germany
Leica Application Suite V.3.8	Leica Microsystems, Wetzlar, Germany
Graphpad Prism 6.0	GraphPad Software, La Jolla, USA
Mircrosoft office 365 15.0	Microsoft Corporation, Washington, USA
SPASS 23	IBM, Ehningen, Germany
ImageJ 1.8.0	National Institutes of Health, Bethesda, USA

Table 3.2 Software

3.1.3 Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM)	Gibco® by life technologies™, CA, USA
Minimum Essential Media (MEM)	Gibco® by life technologies™
Roswell Park Memorial Institute 1640 Medium (RPMI)	Gibco® by life technologies™
MEM Non-essential Amino Acid Solution (100×)	Gibco® by life technologies™
Fetal Bovine Serum (FBS)	Biochrom GmbH, Berlin, Germany
L-Glutamine 200 mM	Biochrom GmbH
Penicillin 10000 U/ml Streptomycin 10000 µg/ml	Biochrom GmbH
DPBS (1×) Dulbecco's Phosphate Buffered Saline w/o $CaCl_2$, $MgCl_2$	Gibco® by life technologies™
TrypLE™ Express Stable Trypsin Replacement Enzyme (+) Phenol Red	Gibco® by life technologies™
Dimethylsulfoxid (DMSO) ≥ 99.5 %	Carl Roth GmbH& Co.KG, Karlsruhe, Germany
Acetic Acid	Carl Roth GmbH& Co.KG
Ammonium Persulfate	Thermo Fisher Scientific, Langenselbold, Germany
Ethanol 98%	Carl Roth GmbH& Co.KG
Ammoniumchlorid (NH_4Cl)	Carl Roth GmbH& Co.KG
Glycerine	Carl Roth GmbH& Co.KG
Glycin	Carl Roth GmbH& Co.KG
Gelatin	Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany
Histokitt II	Carl Roth GmbH& Co.KG
Hoechst 33341	Sigma-Aldrich Chemie GmbH

Kaliumchlorid (KCl)	Carl Roth GmbH& Co.KG
Methanol 99.5%	Carl Roth GmbH& Co.KG
Natriumchlorid (NaCl)	Carl Roth GmbH& Co.KG
Natriumdodecylsulfat (SDS)	Carl Roth GmbH& Co.KG
Natriumhydroxid (NaOH)	Carl Roth GmbH& Co.KG
Triton X-100	Carl Roth GmbH& Co.KG
Paraformaldehyde, Granulated	Carl Roth GmbH& Co.KG
Paraformaldehyde Solution, 4% in PBS	Affymetrix, Cleveland, USA
ß-Mercaptoethanol	Merck Millipore, Burlington, USA
Tris Base	Carl Roth GmbH& Co.KG
Antibody Diluent	Zytomed System GMBH，Berlin，Germany
Antibody Diluent	Dako, Glostrup, Denmark
Crystal Violet	Sigma-Aldrich Chemie GmbH
Goat Serum	Dako, Glostrup, Denmark
Vectashield® Hardset Antifade Mounting Medium	Vector Laboratories, Inc. Burlingame, USA
ROTI® Histokitt II Synthetic mounting medium	Carl Roth GmbH& Co.KG

Table 3.3 Chemicals and Reagents

3.1.4 Consumables and Kit

Tissue Culture Dish 100 × 20 mm	Sarstedt, Inc, Newton, USA
Tissue Culture Plate 6-well-plate	Sarstedt, Inc, Newton, USA
Elisa 96-well Microplate	Thermo Fisher Scientific, Langenselbold, Germany
PCR 96-well Plate	Thermo Fisher Scientific
Corning® Costar® Transwell® Cell Culture Inserts	Corning, New York, USA
Glass-Pasteur Pipette 150 mm, 230 mm	Brand GmbH Co, Wertheim, Germany
Cellstar® Polypropylene Tube 15 ml	Greiner bio-one GmbH, Frickenhausen, Germany

Cellstar® Polypropylene Tube 50 ml	Greiner bio-one GmbH
Biosphere® SafeSeal Micro Tubes 1.5 ml	Sarstedt AG & Co. KG, Nuembrecht, Germany
Pipetten-tips Safeguard 1000 µl, 200 µl, 100 µl, 20 µl, 10 µl	Peqlab Biotechnologie GmbH, Erlangen, Germany
Qiagen RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
Ultraview Universal DAB Detection Kit	Ventana Medical Systems, Tucson, USA
First-Strand Synthesis SuperMix	Thermo Fisher Scientific
KAPA SYBR® FAST Master Mix (2X)	KAPA Biosystems, Boston, USA

Table 3.4 Consumables and Kit

3.2 Methods

3.2.1 Patient selection

The study included 415 patients with OPSCC diagnosed in the ENT department of the University Hospital Rechts der Isar, Munich, during a period of 01.01.2001-31.12.2011. The tissue collection was approved by the local ethical committee. Clinical parameters were retrospectively collected: age, sex, local regional metastasis, distant metastasis, TNM-Staging, grading, treatment modalities to follow-up. The OPSCC patients were classified according to the criterion of the 7th AJCC cancer staging system in order to avoid the pre-interpretation in clinical manifestations regarding the HPV status. The patients with lacking data, incomplete staging and interrupted treatment were excluded. 53 OPSCC were randomly selected and analysed.

3.2.2 Immunohistochemistry (IHC)

3.2.2.1 Ventana BenchMark® GX automated staining system

FFPE blocks were received from the Department of Pathology, University Hospital Rechts der Isar, Munich, where the tissues were trimmed, fixed and paraffin-embedded. The embedded tissue was cut with the Microtom® (Thermo Fisher Scientific) into 1.5 µm thick slides and dried at 60 °C in an incubator overnight. The staining was done using the Ventana BenchMark® GX automated staining system (Ventana Medical Systems), which is a fully automated slide processing system with a barcode-labeled slide recognition unit, standardized ready-to-use buffers, and automated workflow protocol. FFPE tissue slides transferred onto object slides were heated to 75 °C and washed with EZ® prep solution to remove the paraffin. Heat-induced antigen retrieval was carried out using the cell conditioning solution (CC1, Ventana Medical Systems) for all antibodies. 100 µl of the diluted primary antibody was applied and incubated for a dedicated time (see table below). Then anti-mouse/anti-rabbit isotype secondary antibody with horseradish peroxidase (HRP) was incubated to bind to the primary antibody. The slides were washed with H_2O_2 to reduce background staining by inhibiting endogenous peroxidase activity. Subsequently counterstaining was done using hematoxylin. Slides were mounted with 2 drops Roti®-histokitt II and sealed with a coverslip.

Primary Antibody	**Dilution**	**Company**
CXCR4	1:200	R&D Systems Inc, Minneapolis, USA
CXCL12	1:1000	R&D Systems Inc, Minneapolis, USA
TIMP1	1:500	R&D Systems Inc, Minneapolis, USA
TIMP2	1:500	Biomol GmbH, Hamburg, Germany
MMP2	1:100	DCS Innovative Diagnostik-Systeme, Hamburg, Germany
MMP9	1:1000	Thermo Fisher Scientific, Langenselbold, Germany
PLAU	1:500	US Biological, Boston, USA
p16	1:1	Roche, Basel, Switzerland
Secondary Antibody	**Dilution**	**Company**
Anti-Mouse HRP-linked	1:10000	Cell Signaling Technology, Danvers, USA
Anti-Rabbit HRP-linked	1:5000	Cell Signaling Technology, Danvers, USA

Table 3.5 Antibodies

3.2.2.2 Quantification of IHC

Evaluation of the staining was supervised by two pathologists. The evaluation was done using the microscope Leica DMI 6000B Leica DFC 425C (Leica, Wetzlar, Germany). Micrographs were captured with Leica Lab suite software (Version 3.8). The immunoreactive scoring system for immunostaining assessment of tumor cells was used.

Immunoreactive score [SI±PP]		SI (0−3)			
		Negative (0)	Weak (1)	Moderate (2)	Strong (3)
PP [0−4]	Negative (0)	0	1	2	3
	≤10% (1)	1	2	3	4
	11%−50% (2)	2	3	4	5
	51%−80% (3)	3	4	5	6
	≥81% (4)	4	5	6	7

Table 3.6 Immunoreactive score

SI, staining intensity; PP, the percentage of positive cells.

3.2.3 Cell culture

3.2.3.1 Cultivation of head and neck squamous cell carcinoma cancer cell lines

The Cal-27, UM-SCC-111 and UPCI-SCC-154 cell lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), the UD-SCC-2, UD-SCC-4, UD-SCC-5, UD-SCC-6, UD-SCC-7 cell lines from the University of Duesseldorf (Department of Otorhinolaryngology, Duesseldorf, Germany), VU-SCC-147 cell line from VU University Medical Center Amsterdam (Department of Clinical Genetics, Amsterdam, Netherlands), and SAS cell line from JCRB cell bank (Osaka, Japan). HPV-16 positive HNSCC cell lines are UD-SCC-2, UPCI-SCC-154 and VU-SCC-147. The culture medium of these cell lines are as follow: Dulbecco's Modified Eagle Medium (1× DMEM) with 10% fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml penicillin for UD-SCC-4, UD-SCC 5, UD-SCC 6, UD-SCC 7, UM-SCC-111, Cal-27, VU-SCC-147 and SAS; Minimum Essential Media (MEM) with 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 1% non-essential amino acid (100×) for UPCI-SCC-154; RPMI Media 1640 with 10% FBS, 2 mM glutamine, 100 U/ml penicillin for UD-SCC-2. Cell lines were cultured under standard cell culture conditions at 5% CO_2 and 37°C in the CO_2 incubator and grown to 70−90% confluency.

Cell Line Name	Disease	HPV Status	Research Resource Identifiers (RRID)
UD-SCC-2	Hypopharyngeal Squamous Cell Carcinoma	HPV16+	CVCL_E325
UPCI-SCC-154	Tongue Squamous Cell Carcinoma	HPV16+	CVCL_2230
VU-SCC-147	Floor of Mouth Squamous Cell Carcinoma	HPV16+	CVCL_L895
UD-SCC-4	Oropharyngeal Squamous Cell Carcinoma	–	CVCL_E327
UD-SCC-5	Laryngeal Squamous Cell Carcinoma	–	CVCL_L548
UD-SCC-6	Oropharyngeal Squamous Cell Carcinoma	–	CVCL_M120
UD-SCC-7A	Oropharyngeal Squamous Cell Carcinoma	–	CVCL_M118
SAS	Tongue Squamous Cell Carcinoma	–	CVCL_1675
UM-SCC-111	Tongue Squamous Cell Carcinoma	–	CVCL_0C74
Cal-27	Tongue Squamous Cell Carcinoma	–	CVCL_1107

Table 3.7 HNSCC cell lines

3.2.3.2 Thawing of cells

The cells were stored in cryo tubes in liquid nitrogen. To thaw them, the cryo tubes were incubated in a water bath at 37 °C. After the cell suspension had liquefied, they were transferred in a 15 ml falcon containing 10 ml medium. After re-suspending, this cell suspension was pipetted into a 10 cm cell culture dish and incubated overnight in a CO_2 incubator. The medium was changed the next day.

3.2.3.3 Splitting cells

Once the cells in the cell culture dishes reached about 80% confluency (usually after 2 to 3 days), the cells were split. Therefore, the used medium was completely aspirated, and the cells were rinsed with 5 ml of PBS. Afterwards the cells were incubated for 5 min with 2 ml trypsin in the incubator until the cells detached from the cell culture dish. 5 ml of fresh medium was pipetted in culture dish and the cell suspension was transferred to a 15 ml falcon. There,

the cells were resuspended thoroughly. According to the split ratio, adequate cell suspension was taken into a new 10 cm cell culture dishes with fresh medium.

3.2.3.4 Medium change

A medium change took place whenever the required confluence was not attained. Therefore, the used medium was completely aspirated and replaced by 7 ml fresh medium.

3.2.3.5 Freezing cells

Cells with approximately 70−80% confluency were detached as described in 3.2.3.3, Trypsinized cells were transferred into a 15 ml falcon and centrifuged in 2000 rcf for 2 min. The cell pellet was resuspended with 3 ml freezing medium consisting of FBS and 5% DMSO. 1 ml of this cell suspension was pipetted into 1.5 ml cryo tube. The cells were frozen at −80 °C overnight, which allowed a gradual reduction of the temperature. Afterwards the cryo tubes were placed in liquid nitrogen.

3.2.4 Real-time polymerase chain reaction (qPCR)

3.2.4.1 RNA isolation

The RNeasy® mini kit (Qiagen, Hilden, Germany) was used for isolation of RNA from HNSCC cells according to the manufacturer's instructions. When cells reached 70-80% confluency they were washed with PBS and lysed in 500 µl of RLT buffer supplemented with 1% β-mercaptoethanol by incubating for 10 minutes. Then the lysates were transferred to a reaction tube and 1 volume of 70% Ethanol was added to homogenize the lysate. The lysate was mixed by pipetting and 700 µl were transferred to a RNeasy® spin column placed in a 2 ml collection tube. The columns were centrifuged at 12000 rpm for 15 s and the flow-through was discarded. To wash the spin column membrane, 700 µl RW1 buffer was added, centrifuged at 12000 rpm for 15 s and the flow-through was discarded. Two additional washing steps were performed using 500 µl RPE buffer. Centrifugation was performed as described above. After last washing step, the columns were centrifuged for 2 min. RNA was eluated into a fresh 1.5 ml tube with 150 µl RNase-free water by centrifugation at 12000 rpm for 1 min. RNA concentration was measured with the Nanodrop ND1000 spectrophotometer. Absorbance

ratios at A260/280 nm and 260/230 nm were detected to determine RNA concentration and purity. RNA samples were stored at −20°C until further use.

3.2.4.2 cDNA synthesis

Reverse transcription of RNA was done using the First-Strand Synthesis SuperMix Kit according to the manufacturer's instructions. First, the RNA mixes were prepared by mixing 1µl random hexamer primers (50 ng/µl); 250 ng total RNA and 1 µl 10 mM dNTP and adjusted with DEPC-treated H_2O to a total volume of 14.5 µl in a 1.5 ml PCR reaction tube. The reaction tubes were immediately transferred to a heating block (5 min, 65 °C), then transferred on ice. 7 µl of Reverse Transcriptase Mixes was added into the reaction tube and incubated for 2 min at 37 °C.

Reverse Transcriptase Mix: 4 µl 5×First-Strand Buffer
2 µl 100 mM DTT
1 µl Aqua dest.

Subsequently, 1 µl M-MLV Reverse Transcriptase was added into the reaction tubes. The cDNA synthesis mix was incubated 10 min at RT and then 50 min at 37 °C. For the inactivation of enzyme, the tubes were incubated for 15 min at 70 °C. The cDNA was diluted with DEPC-treated H_2O water in the ratio 1:2 and stored at −20 °C until further use.

Primer	**Sequence (5'−>3')**
CXCR4	Forward primer GAGGAGTTAGCCAAGATGTGACT
	Reverse primer AGTAGTGGGCTAAGGGCACA
CXCL12	Forward primer TGAGCTACAGATGCCCATGC
	Reverse primer GCGGAAAGTCCTTTTTGGCT

3.2.4.3 Quantitative real-time polymerase chain reaction (qRT-PCR)

The qRT-PCR applied to the quantitative establishment of mRNA amount for expression analysis which used fluorescent probes specifically. The target RNA sequence was reverse transcribed into cDNA of the target RNA, the cDNA is subsequently amplified through

a DNA polymerase, nucleotides, and primers that function as the complementary of target DNA sequence. KAPA SYBR® FAST qPCR Master Mix (2X) kit was used as a dye for the quantification of double stranded cDNA in methods of quantitative RT-PCR by fluorescence signal intensity. As SYBR Green dye binds to double-stranded DNA, the intensity of the fluorescence increases. The ultimate amount of DNA was evaluated by the intensity of fluorescence. Samples were amplified in a single MicroAmp Optical 96-well reaction plate (PE Applied Biosystems). The 25 µl qPCRs reaction mix was prepared according to the KAPA SYBR® FAST qPCR Master Mix (2X) manufacturer´s instructions (see below). 2 µl of cDNA was used for per reaction. Each reaction ran in triplicate. For each qRT-PCR a negative control, in which cDNA was replaced by water, was carried out. The qPCR cycles were set as follow: one initial denaturation step (3 min at 95 °C), 45 amplification steps consisting of denaturing (15 s at 94 °C), annealing (15 s at 55−65 °C), and elongation (30 s at 70 °C) followed by a fluorescence recording, and a final melting curve (65−96 °C continuous fluorescence recording).

1× qRT-PCR reaction (25 µl): 12.5 µl KAPA SYBR® FAST Master Mix (2X)
1 µl Primer forward (10 µM)
1 µl Primer forward (10 µM)
8.5 µl RNase-free water
2 µl cDNA (6.25 ng/µl)

PCR-programme:

	Temperature	**Time**	
	95 °C	3 min	Denaturing
45×	94 °C	15 s	Denaturing
	55 °C − 65 °C	15 s	Annealing
	70 °C	30 s	Elongation
	65 °C − 96 °C	0.05 °C per 5 s	Melting Curve

Table 3.8 PCR-programme

3.2.4.4 Final qPCR evaluation method

Quantitative mRNA expression analysis was performed using the comparative threshold cycle ($2^{-\Delta\Delta Ct}$ method). The cycle in which fluorescence can be detected is termed quantitation cycle (Ct for short) and is the basic result of qPCR: lower Ct values mean higher initial duplicate numbers of the target gene. Ct values of target genes were normalized to respective Ct values of the control gene GAPDH (Cat. No. QT01192646, Qiagen, Hilden, Germany):

$$\Delta Ct = Ct^{\text{target gene}} - Ct^{GAPDH}$$

Then the relative expression was calculated by normalizing the ΔCt to a cell line, which was set as control, to compare them against each other:

$$\Delta\Delta Ct = \Delta Ct^{\text{ sample}} - \Delta Ct^{\text{control}}$$

$$\text{relative expression} = 2^{-\Delta\Delta Ct}$$

3.2.5 Immunofluorescence

The cells (5×10^5/well) were seeded onto the 0.1% gelatin coated microscope slides in 4 wells plates at 37 °C incubator. After 48 h incubation, 200 µl of 4% Paraformaldehyde/1×DPBS (Affymetrix, Cleveland, USA) was applied for fixing the slides for 20 min incubation at room temperature. Subsequently, 0.5% Triton X-100 in PBS was used to achieve cellular penetration. After 5 mins incubation with 200 µl of 50 mM NH_4Cl/1×DPBS, slides were blocked with 200 µl of 5% normal goat serum/1×DPBS for 30 min incubation. Next, primary antibody against CXCR4 (R&D, Minneapolis, USA) (1:100 dilluted with Antibody Diluent) was added and incubated at 4 °C overnight. The slides were incubated with secondary antibody Cy3™ goat anti-rabbit (H+L) (Invitrogen, California, USA) (1:100 dilluted with Antibody Diluent) at room temperature for 1 h. Subsequently, DNA staining was done by 1 µg/ml Hoechest (Thermo Scientific, Massachusetts, USA) (2 mg/ml diluted in DPBS) and incubation for 5 minutes in a light resistant container. The slides were washed with 1×DPBS 3 times after each treatment. Then the slides were fixed by Vectashield® antifade mounting medium. Images were captured with a Leica microscope imaging system. The quantification of cellular fluorescence in immunofluorescence was performed by ImageJ 1.8.0 software (USA), the corrected total cell fluorescence (CTCF) = Integrated Density − (Area of selected cell ×Mean fluorescence of background readings).

3.2.6 Crystal violet assay

Cell proliferation was evaluated using a crystal violet assay, in which crystal violet binds to cellular DNA. 5×10^3 cells/well were seeded in six-well plates 24 h before treatment, incubating in 5% CO_2 incubator at 37 °C overnight. Next, cells were treated with AMD3100 (Selleckchem, Munich, Germany) with conditioned dose and incubated in 5% CO_2 incubator at 37 °C for 10 days. 500 µl of 4% formaldehyde were added into each well for 30 minutes incubation at room temperature to fix the cell. Subsequently, 2 ml 0.1% Triton-X-100/PBS was added into each well incubating for 5 min. Next, the plate was gently washed three times with tap water. 2 ml crystal violet (0.04%) was added into each well for 30 minutes incubation on a bench rocker. After being washed three times, the six-well plate was air-dried for at least 1 h at room temperature. Finally, 2 ml SDS (1%) was added into each well for 1 h incubation at room temperature on a bench rocker. 100 µl suspension was transferred into 96-well microplate in triplet. The optical density of cells was measured at 590 nm (OD590) using Multiskan FC Microplate Photometer. After setting the average OD590 of non-stimulated cells to 100%, the value of percentage of stimulated cells was determined.

3.2.7 Migration and chemotaxis assay

The Transwell® model was used to investigate the cell migration and chemotactic motility. This was verified by utilizing an in vitro transwell-assay (8.0 µm pore size, 6.5 mm diameter inserts; Corning® Costar® Transwell® cell culture inserts, Corning Inc., NY, USA). The cells were sown when they reached 60−80% confluency in 10 cm petri dish.

Initially, the Transwell® cell culture inserts were placed in a 24-well plate and coated with 600 µl medium (1% FBS) in the well and 100 µl medium (1% FBS) in the insert for 1 hour in 5% CO_2 incubator at 37 °C. Afterward the trypsinised cells in petri dish were counted automatically with the cell counter (Cellometer Auto T4). Then the cells were transferred into 1.5 ml tube and centrifuged (2 minutes, 2000 rcf, room temperature). After removing the medium in 1.5 ml tube, the cell pellet was resuspended in medium containing 1% FBS (the volume of medium was dependent on the amount of cell to reach the final concentration of 5×10^5 cells/ml). After removing the medium in the chamber, 100 µl cell suspension (5×10^4 cells) was pipetted into the insert and 600 µl medium with/without recombinant CXCL12 (PromoCell GmbH, Heidelberg, Germany) was added into the lower compartment of Transwell® chamber.

The 24-well Transwell® plate was incubated in 5% CO_2 incubator at 37 °C for 24 h. Following incubation, the inserts were washed with PBS 3 times. Cotton swab was chosen to clean up the cells from the seeding side of the Transwell® membrane, whirling gently on the membrane to avoid destroying the membrane. The membrane was cut off by scalpel along with the edge of the inserts. The membrane was then transferred into a well of a 6-well plate. Fixation of the cells on the membrane was applied by 4% formaldehyde within a 5-minute incubation at room temperature. After 3 times PBS washing 0.5% Triton X-100 in PBS was applied to achieve cellular penetration. DNA staining was done by adding 1 µg/ml Hoechest (2 mg/ml diluted in PBS) and incubation for 5 minutes in a light resistant container. The membrane was washed with PBS 3 times after each treatment. Then the membrane was fixed on the object slides by Vectashield® antifade mounting medium. The cells were counted using the 40× magnification lens of the Leica DFC 425 C microscope. The cells number of each membrane was counted with the help of mechanical counter. Each membrane was counted 3 times and the average was used for evaluation.

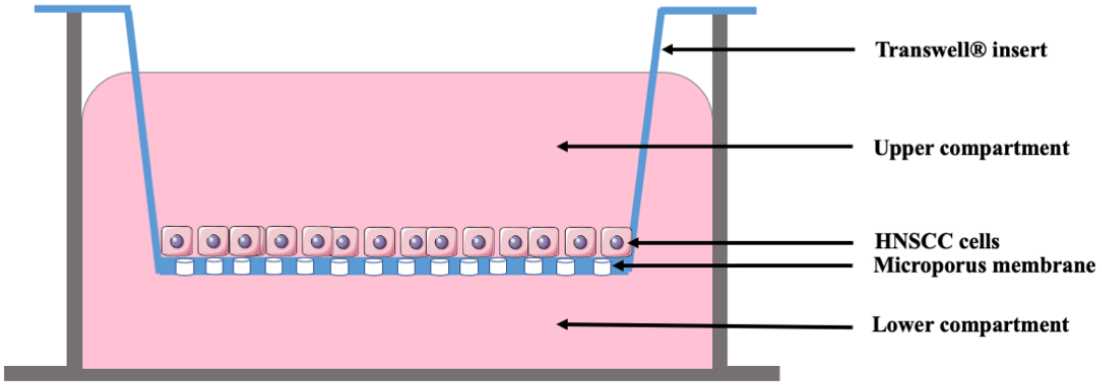

Figure 3.1 Schematic representation of the Transwell® model in migration/chemotaxis assay

The Transwell® model consists of two compartments separated by a microporous membrane. The HNSCC cells were seeded on the membrane of the upper chamber. The medium containing chemoattractant is added into the lower compartment.

3.2.8 Statistical analyses

Statistical analysis was performed with SPASS 23 (IBM, Ehningen, Germany) or GraphPad Prism 6.0 (GraphPad Software Inc, CA, USA). All experiments were independently repeated at least three times. The data were presented as mean ± standard deviation (SD). Comparisons between two groups were analysed with student's t test. For pairwise multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison test. Significance is indicated as $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***), while ns indicates non-significant p-value.

4 Results

4.1 Clinical and pathological characteristics in OPSCC patients regarding HPV status

A total of 53 patients with OPSCC patients was randomly selected from the entire cohort of 415 patients, underwent treatment in the Department of Otorhinolaryngology, Technical University Munich (Table 4.1). Among of them, 36 patients presented with HPV positivity and 17 patients presented with HPV negativity classifying by p16 immunohistochemistry analysis in primary tumor. In groups, gender distribution showed no significant difference ($p = 0.78$). At the initial presentation no significant difference of age between OPSCC patients with different HPV status ($p = 0.87$). HPV positive OPSCC were mostly staged as T2 (53%), whereas HPV negative OPSCC presented a proportional T-status distribution. The positivity of lymph node metastasis was high in HPV positive OPSCC (HPV positive/negative: 83%/65%; $p = 0.56$) and manifested a dominant stage N2a/b (67%) metastasis. Distant metastasis involvement in patients of HPV negative OPSCC exclusively occurred (6%). The majority of patients (HPV positive/negative: 75%/71%, $p = 0.87$) underwent surgical approaches with an adjuvant radio-(chemo-)therapy.

	HPV pos.	HPV neg.	p-value
N	36	17	
Age at diagnosis, mean ± SD	61 ± 10	62 ± 11	0.87
Gender, M:F (%)	24:12 (67:33)	12:5 (71:29)	0.78
T status, n (%)			0.90
T1	8 (22)	6 (35)	
T2	19 (53)	5 (29)	
T3	5 (14)	3 (18)	
T4	4 (11)	3 (18)	
N status, n (%)			0.56
N0	6 (17)	6 (35)	
N1	3 (8)	1 (6)	
N2a/b	24 (67)	6 (35)	
N2c	3 (8)	4 (24)	
N3	0	0	
M status			0.33
M0	36 (100)	16 (94)	
M1	0	1 (6)	
UICC stage			0.56
I	1 (3)	2 (12)	
II	4 (11)	1 (6)	
III	3 (8)	3 (18)	
IV	28 (78)	11 (64)	
Therapy			0.87
Surgery only	3 (8)	2 (12)	
Surgery + aC/RT	27 (75)	12 (71)	
p C/RT	6 (17)	3 (18)	

Table 4.1 Depiction of the clinical and pathological characteristics of 53 OPSCC patients

4.2 Expression of CXCL12 is significant high in HPV-related OPSCC primary tumor

The expression of CXCR4-CXCL12 axis related proteins were assessed immunohistochemically in 53 OPSCC tissues (Figure 4.1). Specifically, CXCR4 protein was expressed in primary tumor of the HPV positive OPSCC tissues, predominantly in the membrane of tumor cells. The significantly higher expression of CXCL12 was demonstrated in HPV-positive primary OPSCC ($p = 0.027$). Immunohistochemistry of MMP2, MMP9, TIMP1, TIMP2, CXCR4 and PLAU did not show statistical differences between HPV positive and negative OPSCC primary tumors, nevertheless the high expression of immunohistochemical CXCR4, TIMP2 and PLAU was observed in HPV positive primary tumors.

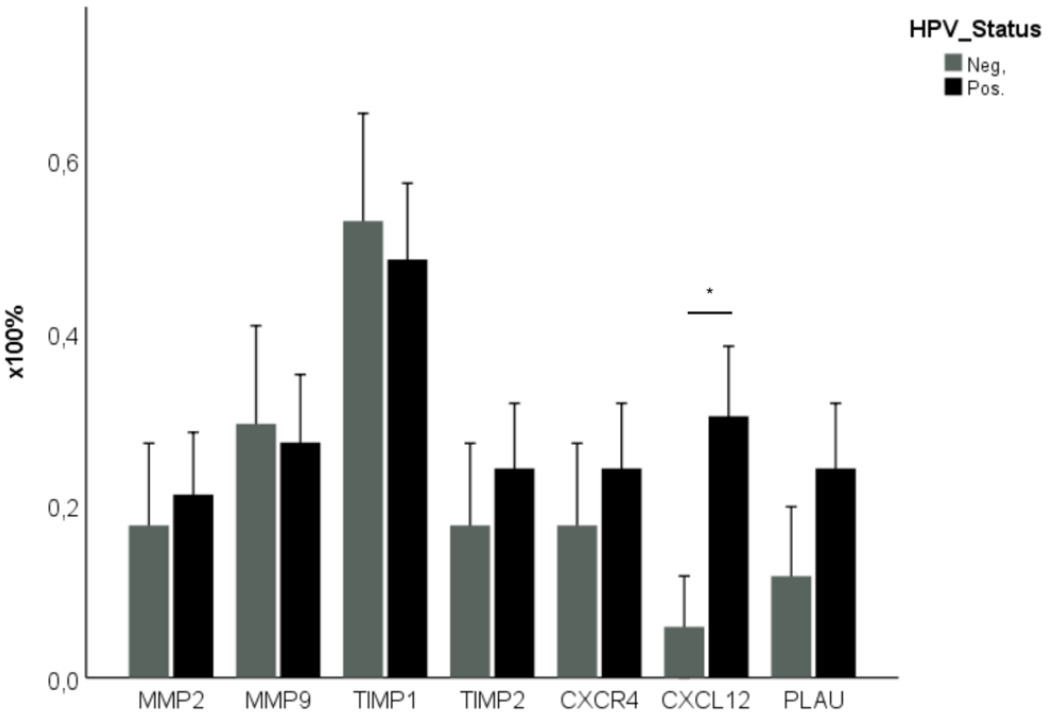

Figure 4.1 Immunohistochemical analysis of CXCR4-CXCL12 axis related proteins expression in OPSCC patients regarding HPV status

(* $p < 0.05$)

4.3 Expression of CXCR4 and CXCL12 is high in HPV-related HNSCC cell lines

To assess the expression of CXCR4-CXCL12 in HNSCC cell lines in vitro, the expression of CXCR4 and CXCL12 was detected in selected HNSCC cell lines by quantitative Realtime-PCR (qRT-PCR) analysis. As shown in Figure 4.2 (A, C), qRT-PCR results showed that CXCR4 was significantly stronger expressed in HPV positive HNSCC cell lines ($p = 0.028$) as compared to HPV negative HNSCC cell lines. Furthermore, significantly higher mRNA expression of CXCL12 in HPV positive cell lines was demonstrated in 10 HNSCC cell lines ($p = 0.015$, Figure 4.2 B, D). To further confirm the specificity of results in protein level, we did immunofluorescence with selected 4 HNSCC cell lines including HPV positive cell lines (UPCI-SCC-154 and UD-SCC-2) and negative HNSCC cell lines (UD-SCC-5 and UD-SCC-7). Specifically, CXCR4 protein was expressed predominantly in the cell membrane and cytoplasm in both of HPV positive and negative HNSCC cells (Figure 4.3 A). Accordingly, in immunofluorescence staining of CXCR4, the level of cellular fluorescence was analysed, HPV positive cell lines (UPCI-SCC-154 and UD-SCC-2) exhibited significant high CXCR4 in contrast to HPV negative cell lines (UD-SCC-5 and UD-SCC-7) ($p = 0.037$, Figure 4.3 B). Although HPV positive cell lines displayed significantly high intracellular CXCL12 expression in mRNA level, we failed to detect it in cell culture with western blot.

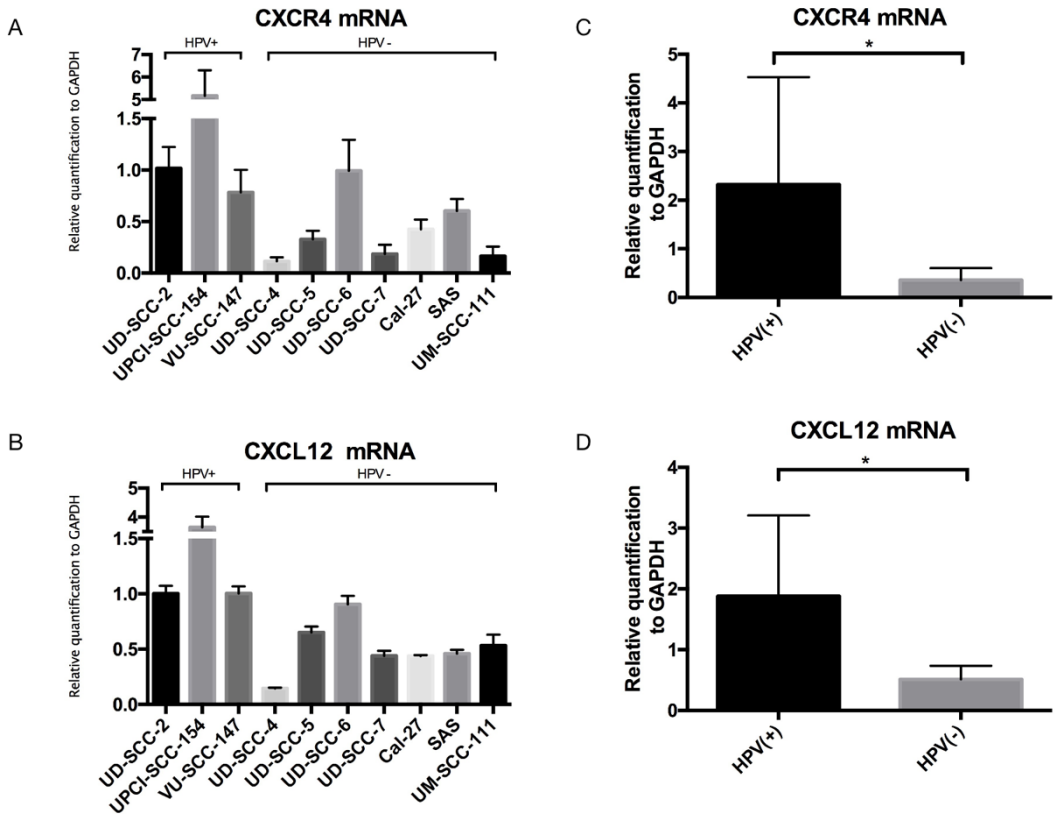

Figure 4.2 qRT-PCR analysis of CXCR4 expression in HNSCC cell lines regarding HPV status

In qRT-PCR, level of relative mRNA expression was normalized to GAPDH. The bars indicated the mean values of three independent experiments. The expression of CXCR4 and CXCL12 mRNA was measured in 10 HNSCC cell lines (A, B). The expression of CXCR4 and CXCL12 in different HPV category of HNSCC cell lines (C, D) (* $p < 0.05$).

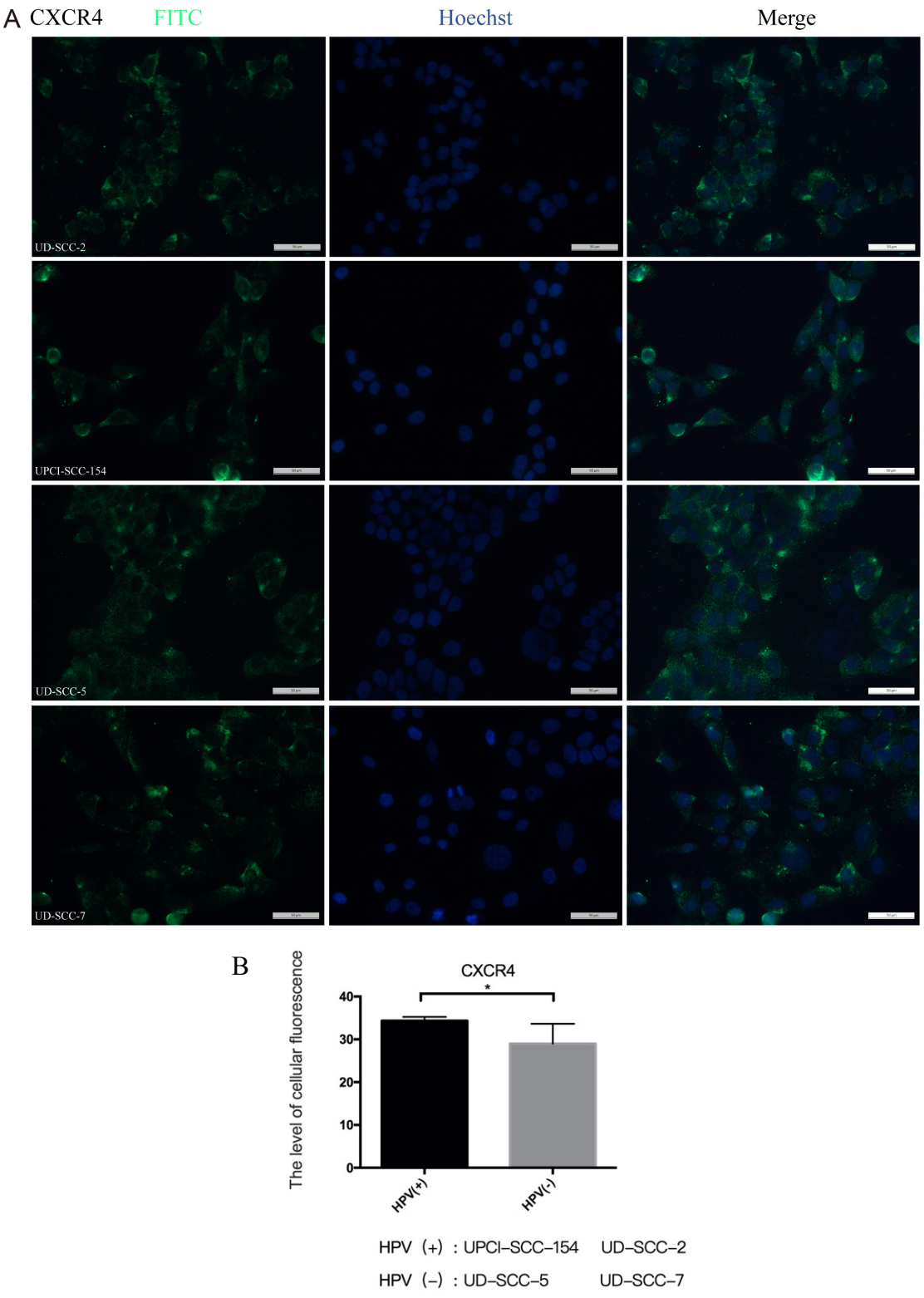

Figure 4.3 Immunofluorescence of CXCR4 in HNSCC cell lines and quantification

Staining of CXCR4 expression (green) by immunofluorescence in HPV positive (UP-SCC-2, UPCI-SCC-154) and negative (UP-SCC-5, UP-SCC-7) HNSCC cell lines. Nuclei (blue) were counterstained with Hoechst (A). Original magnification ×20. Scale bars = 50 µm. Quantification of cellular fluorescence of

CXCR4-staining analyses (B). Images were captured of three random views (mean ± SEM). The error bars show the standard deviation of at least three independent measurements (* $p < 0.05$).

4.4 Involvement of CXCR4-CXCL12 signalling in regulation of HNSCC cell proliferation

To evaluate the involvement of activation of CXCR4-CXCL12 signalling in cell proliferation of HNSCC, cells were treated with AMD3100 (Sellekchem, Munich, Germany), a selective CXCR4 antagonist. Cell proliferation was analysed by the crystal violet staining. A dose range of 0.25 µM to 15 µM of AMD3100 was applied. As shown in Figure 4.4, the proliferation of HPV positive HNSCC cell line UPCI-SCC-154, with high expression of CXCR4 and CXCL12, was significantly inhibited with AMD3100 treatment in a concentration-dependent manner ($p < 0.001$). In HPV negative HNSCC cell line UD-SCC-5, the inhibition in cell proliferation was observed with significant statistical differences after the AMD3100 treatment starting from 1 µM ($p < 0.001$). Furthermore, HPV positive cell line manifested a strikingly reduced proliferation in number of cells in comparison to HPV negative cell line. These results identified the activation of CXCR4-CXCL12 signalling contributing to tumor cell proliferation in HNSCC regarding HPV status.

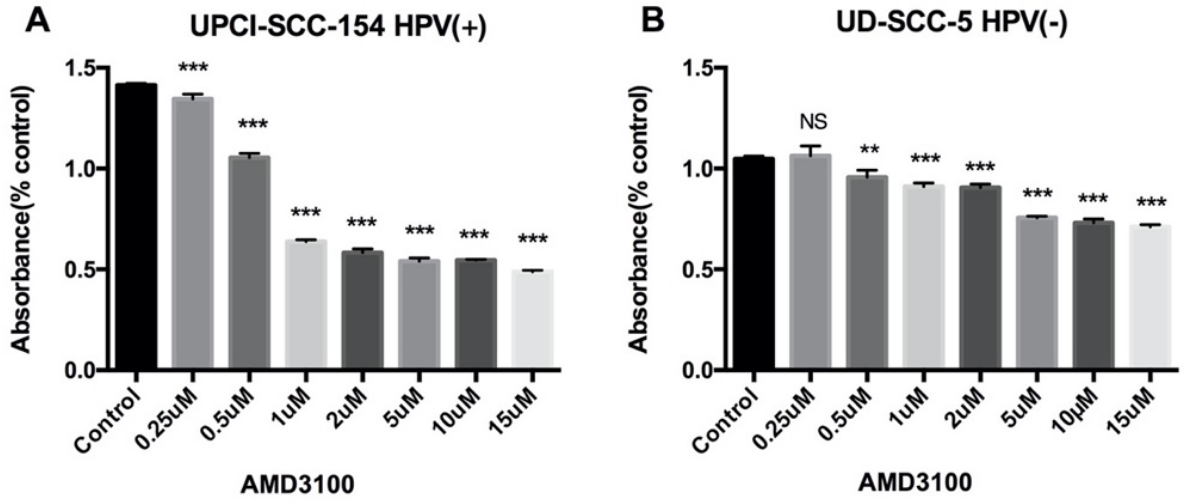

Figure 4.4 Proliferation assay analysis in UPCI-SCC-154 and UD-SCC-5 HNSCC cell line with AMD3100 treatment

HNSCC cells were treated with AMD3100 (concentration from 0.25 µM to 15 µM), in triplicate, and incubated 24 h. AMD3100 significantly inhibited the proliferation of the HNSCC cells compared to the negative control (A, B). Measurement was performed by Elisa reader. ** $p < 0.01$, *** $p < 0.001$, compared with the control group.

4.5 Regulation of CXCR4-CXCL12 signalling in HNSCC cell migration and chemotaxis regarding HPV status

To determine the role of CXCR4-CXCL12 signalling in cancer cell migration and chemotaxis, Transwell® migration/chemotaxis assay was performed. Ichida et al. previously demonstrated that the cell migration induced by CXCL12 was chemotactic instead of chemokinetic (Uchida et al. 2003). In our study, exogenous CXCL12 co-culture did not alter the cell migration considering HPV status in HNSCC cell lines in a serum-conditioned environment (data not shown). The HPV positive UPCI-SCC-154 and HPV negative UD-SCC-5 cell line both showed a significantly increased migration towards fetal bovine serum (FBS), in a concentration-dependent manner from 0.5% to 2% (Figure 4.5 A, B). HPV positive cell lines UPCI-SCC-154 showed a striking increase rate in number of migrated cells.

Considering the manifestation of CXCL12 as a chemoattractant in cancer cells migration, recombinant human CXCL12 was applied in the sub-compartment in chemotaxis assay to evaluate the influence of CXCL12 in the chemotaxis of HNSCC cell lines. HPV negative UD-SCC-5 cell line, which slightly expressed CXCR4 and CXCL12, significantly migrated in number of cells in a condition of increased concentration from 5–100 ng/ml of CXCL12 with a statistical significance ($p < 0.05$, Figure 4.5 C), whereas HPV positive UPCI-SCC-154 cell showed a significantly suppressed migration in a concentration of 100 ng/ml CXCL12 ($p < 0.01$, Figure 4.5 D). These results indicated that the HPV positive cell lines manifested a more aggressive migratory capacity than HPV negative cells as regards CXCR4-CXCL12 expression level. On the contrary, in chemotaxis assay, CXCL12 as a chemoattractant, HPV positive HNSCC cells presented a suppressed mobility despite a high autocrine CXCR4-CXCL12 level.

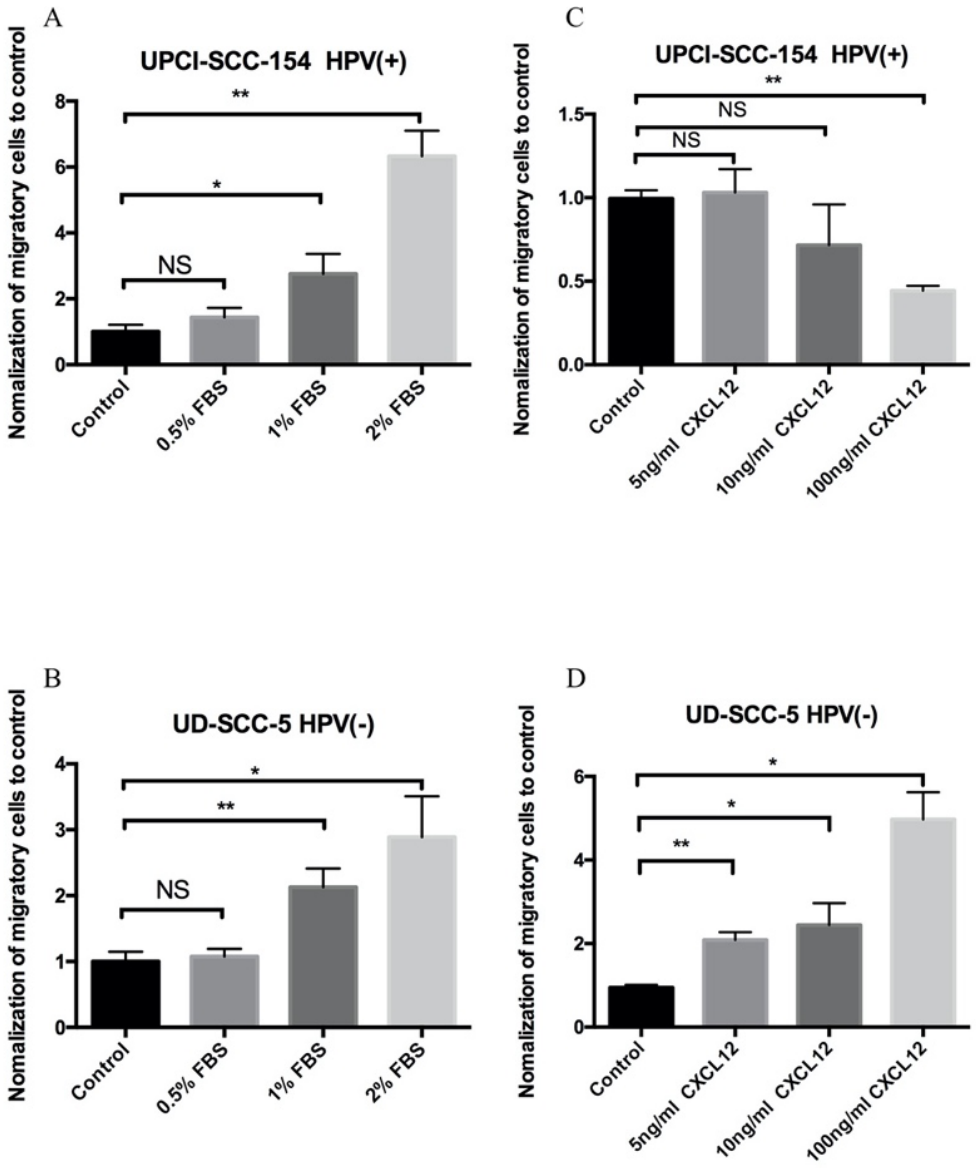

Figure 4.5 Evaluation of CXCR4-CXCL12 in migration and chemotaxis assay of HNSCC cell lines regarding HPV status

HNSCC cells were seeded into the Transwell® insert upper compartment, the FBS or CXCL12 with different concentration was added in the lower compartment. The error bars indicate the standard deviation of the mean. * $p < 0.05$; ** $p < 0.01$; compared with the control group.

4.6 AMD3100 and cisplatin significantly inhibited the migration and chemotaxis of HPV-related HNSCC cell

To assess the effectiveness of an inhibitor on CXCR4-CXCL12 dependent migration due to different HPV status, AMD3100 was used. Under the treatment of AMD3100 (2 μM), HPV positive cell lines (UP-SCC-154 and UD-SCC-2) demonstrated significant inhibition in the migrated cell number as compared to HPV negative cell lines (UD-SCC-5 and UD-SCC-7) either in chemotaxis assay with CXCL12 (100 ng/ml) or in migration assay with FBS (1%) ($p = 0.002$, Figure 4.6 A; $p < 0.001$, Figure 4.6 B).

Cisplatin is a platinum-based first line chemotherapeutic of HNSCC, being applied to combined radiotherapy as well as adjuvant therapy (Fulcher et al. 2018). To investigate whether the impact of the treatment of cisplatin in cell migration is associated with the HPV status and CXCR4-CXCL12 signalling, cells were treated with 1 μM cisplatin (Teva, Ulm, Germany) in Transwell® assay and incubated for 24 hours. In migration assay with exclusive FBS (1%) in sub-compartment, both of HPV negative and positive cell lines exhibited a suppressed response in migration with cisplatin treatment. Moreover, significant differences of drug-susceptibility were not observed between HPV negative and HPV positive cell lines (Figure 4.6 C). However, in chemotaxis assay with chemoattractant CXCL12 (100 ng/ml), HPV positive cell lines showed a higher response with suppression of migratory cells after cisplatin treatment in comparison to HPV negative cell lines ($p = 0.029$, Figure 4.6 D). Furthermore, in a combined treatment of cisplatin and AMD3100, a significantly preferable therapy-susceptibility in HPV positive cell lines was demonstrated in chemotaxis assay as well. ($p = 0.048$, Figure 4.6 F).

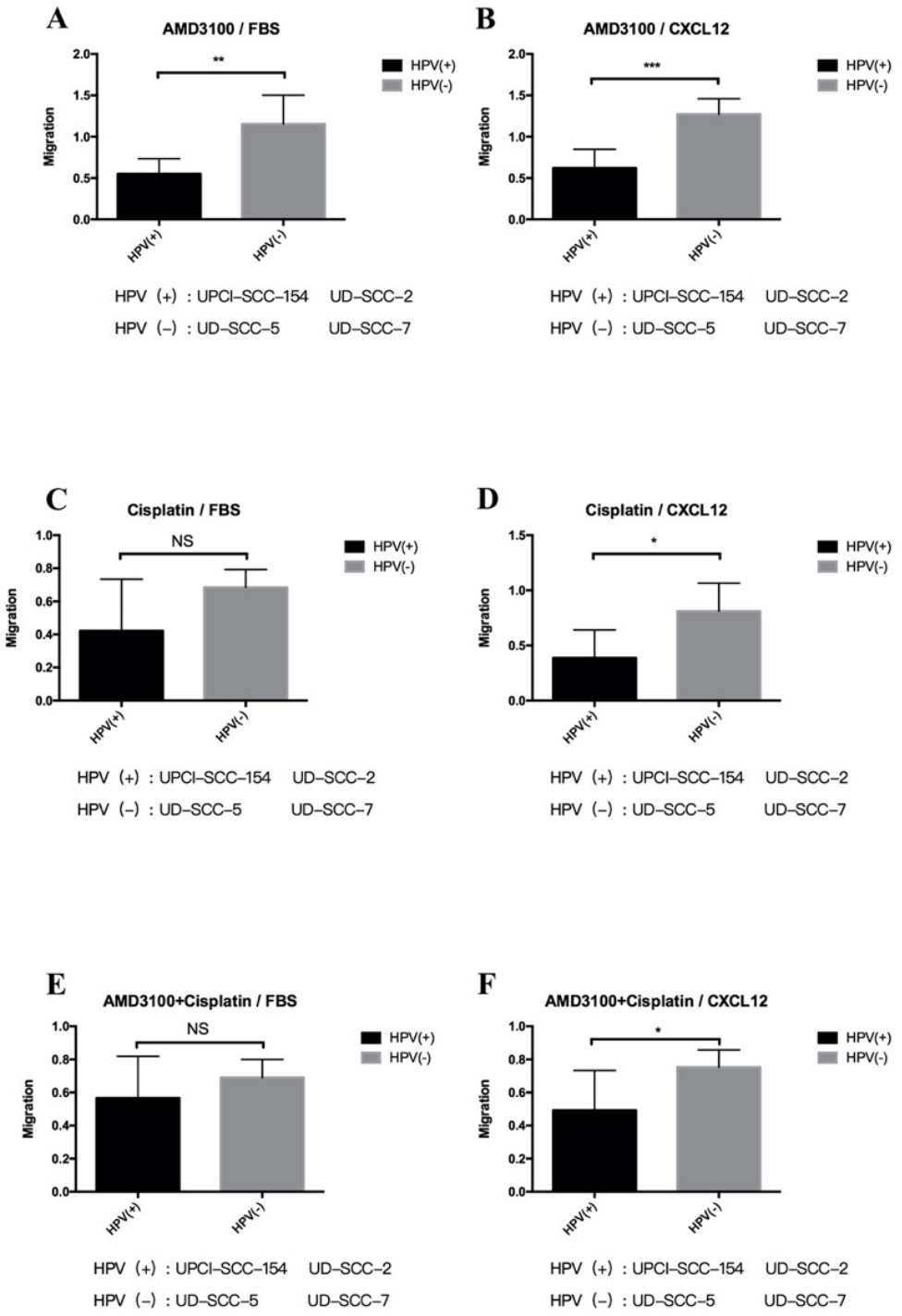

Figure 4.6 Impact of cisplatin and AMD3100 in migration/chemotaxis assay in HNSCC cell lines regarding HPV status

HNSCC cells were treated with AMD3100 (2 µM) and cisplatin (1 µM), individually or combined, before being seeded into Transwell® inserts compartment, the FBS (1%) or CXCL12 (100 ng/ml) was added into the lower compartment. The ratio of relatively migrated cell number was normalized to control group.

5 Discussion

HNSCC, as one of the most common malignancy, is considered a predominantly locoregional disease despite up to 1/4 patients suffering from a distant metastasis (Vokes, Cohen, and Grandis 2008). Tobacco and alcohol exposure are traditionally considered as the risk factor of HNSCC. In the past 30 years, a significant increased incidence of HPV-associated OPSCC among younger patients (<45 years) was disclosed (Andersen et al. 2014; Liu et al. 2016). The poor prognosis mainly attributed to the local recurrence, distant metastasis and therapy resistance in HNSCC. HPV status of OPSCC has been shown to significantly associate with overall survival and event-free survival (Sedaghat et al. 2009; Ang, Harris, Wheeler, Weber, Rosenthal, Nguyen-Tan, et al. 2010). HPV-associated OPSCC is staged differently as compared to non-HPV-associated OPSCC according to the AJCC 8th edition Staging Manual (Lydiatt et al. 2017).

In recent years, CXCR4-CXCL12 signalling was demonstrated to play a key role in HNSCC and several other solid tumor entities (Sun et al. 2010; Balkwill 2004). A prominently predictive feature of the CXCR4-CXCL12 axis was established in HNSCC patients in survival and locoregional and systematic recurrence (Knopf et al. 2017; Yin and Gao 2007). The activation of CXCR4-CXCL12 axis interplaying with PI3K/Akt and ERK/MAPK signalling pathways to promote tumor cell invasion, metastasis and chemotaxis in HNSCCs has been fundamentally established (Teicher and Fricker 2010a).

Since the CXCR4-CXCL12 signalling was implicated in the oncogenesis of HPV, the potential characteristics of CXCR4-CXCL12 in HPV associated HNSCC has not been disclosed properly (Chow et al. 2010; Meuris, Gaudin, et al. 2016).

5.1 CXCR4-CXCL12 axis expression in HNSCC regarding HPV status

OPSCC, as a subset of HNSCC, includes cancers arising from the palatine tonsils, base of tongue, soft palate, and the lateral and posterior pharyngeal walls. HPV-associated OPSCC comprises approximately 25% of HNSCC (Tanaka and Alawi 2018). Up to 45.8% (95% CI 38.9–52.9) of OPSCCs are high-risk HPV attributable from systematic review and meta-analysis with a global pooled estimate of OPSCC (Ndiaye et al. 2014). HPV-associated OPSCC

was found to have more favorable prognosis and better treatment response than conventional smoking- and alcohol-related HNSCC, in contrast it displayed an aggressive clinical phenotype with a high proportion of lymph node positivity instead small T status (Ang, Harris, Wheeler, Weber, Rosenthal, Nguyen-Tân, et al. 2010; Benson et al. 2014). In our cohort, randomly selected HPV positive OPSCC patients accounted for about 68%. The epidemiological profile of HPV-associated OPSCC is substantially distinct with a preference of being young, white, male and limited or no exposure to tobacco from reported studies (Sandulache et al. 2019). Nevertheless, statistical difference was not established in age, gender and other clinical-pathologic parameters at diagnosis from our study with OPSCC regarding HPV status. Clinically, HPV-associated carcinoma tended to display a small T status with an advanced nodal status. From our cohort, the rate of lymph node metastasis was presented with 83% in HPV-associated OPSCC, with a predominant stage as N2a/b (67%) according to the 7th AJCC staging system. In post-radiotherapy OSCC patients, despite a similar long-term distant control regarding HPV status, metachronous second primaries were found in 6% HPV positive cohort and 16% in the HPV negative cohort ($p = 0.003$) after longer intervals. In addition, HPV positive OSCC had a higher incidence of multiple organ dissemination (Huang et al. 2012). Interestingly, the rate of distant control significantly decreased and tended to be stable after diagnoses at 2 years in non-HPV-associated OSCC, however in HPV-associated OSCC distant control achieved relatively stable until 5 years (O'Sullivan et al. 2013). Even though current therapeutic regimens were discussed to be shifted considering the improved survival of HPV-associated OPSCC, HPV positive OPSCC tumors showed heterogeneity in response to the treatments and appeared to have a distant late-metastasis (Trosman et al. 2015). Ang et al. demonstrated that HPV-associated tumors had a 51% reduced risk in relapse or death in comparison with HPV negative tumors with OSCC (Ang, Harris, Wheeler, Weber, Rosenthal, Nguyen-Tân, et al. 2010).

CXCR4-CXCL12 regulated signalling pathways were demonstrated to participate in the regulation of a variety of onco-pathogenetic outcomes, outstandingly in tumor cell survival, invasion and metastasis (Scala 2015). As CXCR4-CXCL12 signalling was disclosed in involvement of the oncogenesis of HPV, CXCR4 was demonstrated as a pivotal susceptibility factor in HPV-associated pathogenesis (Chow et al. 2010; Meuris, Carthagena, et al. 2016). CXCR4, as CXCL12 receptor, with an undetectable feature in normal head and neck epithelium showed high expression in primary HNSCC from collected observations (Albert et al. 2013). In our analysis, CXCR4 predominantly expressed in tumor cell membrane of primary OPSCC patient regardless of the HPV status, which was consistent with other collected studies

in HNSCC (Clatot et al. 2015; Rave-Fränk et al. 2016; Al-Jokhadar, Al-Mandily, Zaid, and Azar Maalouf 2017; De-Colle et al. 2018). Moreover, nuclear staining of CXCR4 was observed in tongue squamous cell carcinoma (Ishikawa et al. 2006a) and in metastatic renal cell carcinoma instead primary renal cell carcinoma (Wang et al. 2009). Nevertheless, the nuclear location of CXCR4 in our study was not found. In our previous study, CXCR4 positivity was demonstrated to associate with an increased risk of tumor recurrence-related death in HNSCC (Knopf et al. 2017). Likewise, other investigations that the patients relating to overexpressed CXCR4 had significantly worse 5-year overall survival were assessed in HNSCC (De-Colle et al. 2018; Almofti et al. 2004). CXCL12, as CXCR4 exclusive chemokine ligand, plays an indispensable role in CXCR4-CXCL12 signalling regulated tumor behavior due to its heterogeneous expressive distribution.

The expression of CXCL12 was revealed to express in primary cancer cells as well as in stromal cells surrounding cancer cells and the metastatic lymph nodes from reported studies in HNSCC patients (Tan et al. 2008; Clatot et al. 2015; Uchida et al. 2007). Since the specific expression of CXCL12 was demonstrated in low-grade HPV-induced lesions (Balabanian et al. 2005), the co-expression of CXCL12 and $p16^{INK4a}$ had been established in dysplastic epidermal keratinocytes in WHIM syndrome regarding gain-of-function mutated CXCR4 gene (Chow et al. 2010). After the correlation between CXCR4-CXCL12 and high-risk HPV16 or HPV18 induced carcinogenesis by interferring E6 and E7 oncoproteins expression was found, CXCR4-CXCL12 signalling as a susceptible factor in HPV pathogenesis was subsequently confirmed (Meuris, Carthagena, et al. 2016). In our present study, the significantly high expression of CXCL12 was demonstrated in HPV positive OPSCC primary tumor. To our knowledge, our finding is the first one which demonstrated a high expression of CXCL12 in HPV positive OPSCC primary tumor in comparison to negative counterpart.

Clinically, the evaluation of CXCL12 expression in survival of HNSCC showed some discrepancies among studies on account of the varied detection methods or the tumor location in HNSCC (Albert et al. 2013). De-Colle et al. demonstrated a correaltion between the expression of intracellular CXCL12 and the worse loco-regional control (LRC) in HNSCC patients as well as in the HPV16 negative subgroup of HNSCC patients (De-Colle et al. 2017). Following study revealed that patient with high expressed intracellular CXCL12 and CXCR4 had a relatively higher loco-regional recurrency after RT-CT in HNSCC (De-Colle et al. 2018). Moreover, Fraenk et al. established a significant correlation between the expression of CXCR4 and CXCL12 respectively and HPV/$p16^{INK4A}$ positivity in inoperable HNSCC patients by means of immunohistochemical staining (Rave-Fränk et al. 2016). Considering the

manifestation of CXCL12 in promoting tumor cell adhesion to ECM proteins (fibronectin and collagen) in HNSCC, MMPs, as the regulator in degradation of ECM, gained considerable focuses in relation to CXCR4-CXCL12 axis (Samara et al. 2004).

MMPs, especially MMP9, was demonstrated to associate with invasion and metastatic spread considering its high expression in distant metastasis in HNSCC (Dunne et al. 2005). The regulation in migration and invasion of OSCC via CXCR4 depending on both MMP9 and MMP13 expression indicating the involvement of MMPs in downstream of CXCR4-mediated signalling (Yu et al. 2011). Furthermore, a CXCL12-mediated secretion of MMP9 by way of activation of the ERK-1/2 signalling pathway was found to promote cell adhesion in HNSCC (Samara et al. 2004). Recently, a report pointed out a significantly decreased concentration of MMP9 and MMP9/TIMP1 ratio from serum of HNSCC patients after surgery with lymph node involvement, the imbalance between MMP9 and TIMP1 was considered to be a sign of metastatic spread in HNSCC (Stanciu et al. 2017). In addition, the participation of the uPA/PLAU in the migration and invasion of cancer cells via activation of MMP2 and MMP9 implied its potential role in CXCR4-CXCL12 signaling (Legrand et al. 2001; Zhan et al. 2020). Considering that the infiltrating leukocytes in HPV-16 induced carcinogenesis had the capacity to secrete MMP9 (Balabanian et al. 2005), MMP2, MMP9, TIMP1, TIMP2 and PLAU were taken into our IHC analysis. The statistical difference was not found in MMPs proteins and their inhibitors such as MMP2, MMP9, TIMP1 and TIMP2 as well as PLAU between the HPV positive and negative group.

To further establish the expression of CXCR4-CXCL12 in vitro, quantitative real-time PCR analysis was accomplished in HNSCC cell lines. High expression of CXCR4 and CXCL12 in mRNA level was demonstrated in HPV-associated HNSCC cell lines. Immunofluorescence staining further visualized the CXCR4 expression in HPV-associated HNSCC cell lines. Quantification analysis of cellular fluorescence revealed the high expression of CXCR4 in HPV-assciated HNSCC cell lines. The similar results of CXCR4 staining in HPV positive OSCC cell lines were presented by Jungbauer et al., but the expressive quantification of protein was not performed therein (Jungbauer et al. 2017). Although the CXCR4 expression was not shown statistical difference between HPV positive and negative primary OPSCC from our results, we considered that in vitro cell experiments the statistical significance of CXCR4 could be properly performed owing to the better sensitivity of qRT-PCR and immunofluorescence.

5.2 The CXCR4-CXCL12 signalling in cell proliferation of HNSCC

Well-documented investigations pointed out that CXCR4-CXCL12 signalling activation contributes to the development of tumor growth, invasion, migration and cell cycle progression in HNSCC (Berning et al. 2018; Kijowski et al. 2001; Faber et al. 2007). The involvement of CXCR4-CXCL12 signalling in HNSCC cell proliferation by down-regulating the CXCR4 in cells has been discussed by Hong et al (Hong et al. 2009). Nevertheless, the role of CXCL12 in the proliferation of HNSCC cells remains controversial. Due to the undetectable CXCL12 in OSCC cell lines, reported by Uchida et al, the exogenous stimulation of CXCL12 failed to activate the growth of HNSCC cells, additional growth inhibition on the cell at a high concentration of CXCL12 was observed in high-CXCR4 expressing cell lines (Uchida et al. 2003). Furthermore, Jungbauer et al. found no significant impact from the stimulus of exogenous CXCL12 on cell proliferation of either HPV positive or negative HNSCC cells despite a differentiated CXCR4 expression level (Jungbauer et al. 2017). Nevertheless an observation from Katayama et al. demonstrated that exogenous CXCL12 enhanced the proliferation in CXCR4-expressing HNSCC cell line (Katayama et al. 2005). In addition to that, the strengthened colony formation was observed in an overexpressed CXCL12 transfected OSCC cells with a high CXCR4 expression, even though the CXCR4 protein on the cell surface was not changed (Uchida et al. 2007). Collectively, the autocrine intracellular CXCL12 has a certain function of regulating cell proliferation with CXCR4 instead paracrine CXCL12 in HNSCC. In our study, AMD3100, as CXCR4 selective antagonist, significantly inhibited the the cell proliferation with regard to the detectable intracellular CXCL12 in both HPV positive and negative cell lines. Our finding indicated that the signalling activation from endogenous CXCL12 with CXCR4 contributes to the HNSCC tumor proliferation.

5.3 The CXCR4-CXCL12 signalling in cell migration/chemotaxis of HNSCC regarding HPV status

Chemokines and their receptors play a key role in regulating metastasis and chemotaxis process. The activation of several downstream signalling pathways via CXCR4-CXCL12 axis potentially facilitates metastatic and chemotactic process including in adherence of tumor cells, angiogenesis, extravasation from blood vessels and cell migration (Scala 2015). Due to the frequently detected CXCL12 in adjacency of cancer cells such as stromal cells and

lymphocytes instead in cancer cells themselves (Clatot et al. 2015; Tan et al. 2008), the CXCL12 concentration gradient existing between metastatic sites and primary tumor was demonstrated as a facilitator in development of metastasis of cancer cells relying on CXCR4-CXCL12 activation, proved by other investigators findings (Uchida et al. 2007; Muller et al. 2001).

Initially low-level expression of CXCL12 was detected in primary tumors with a concordant CXCR4 expression in the patients of OSCC suffered with the development of metastasis (Clatot et al. 2011). Furthermore, Uchida et.al disclosed that the autocrine CXCR4-CXCL12 signalling was proven as a key factor in the regulation of cells distant metastasis in xenograft models despite a facilitation in loco-regional metastasis via paracrine CXCR4-CXCL12 (Uchida et al. 2007). In our migration assay, HPV positive cell lines with high level autocrine intracellular CXCL12 and a concomitant high CXCR4 expression showed aggressive motility towards FBS in comparison with HPV negative HNSCC cell lines. However, the chemotactic motility towards CXCL12 of HPV positive cell lines featured a suppressed motility, in contrast HPV negative HNSCC cell lines showed a dose-dependent chemotaxis to CXCL12. Our finding was consistent with the finding from Jungbauer et al. that HPV negative OSCC cell lines showed an aggressive chemotaxis towards CXCL12, whereas HPV positive cell lines contrary manifested a suppression in chemotaxis assay of CXCL12 (Jungbauer et al. 2017).

Considering the evidence that gain-of-function CXCR4-CXCL12 signalling pathways related to the mutation of CXCR4 in HPV-induced pathogenesis (Meuris, Carthagena, et al. 2016), it might be postulated that CXCR4-medicated pathogenesis in HPV-associated tumor accounted for the desensitization of HNSCC cells in CXCL12-mediated chemotaxis. In addition, a finding from Chow et al. demonstrated that the strong staining of CXCL12 from HPV-18 human keratinocytes cell line displayed a significant fade away in the presence of excessive exogenous CXCL12 in immunofluorescence staining in WHIM syndrome (Chow et al. 2010). We hereby extrapolated basing on these observations that in HPV positive cell lines autocrine endogenous CXCL12 expression might be impeded when cells were exposed to the external environment with exogenous CXCL12, consequently the inactivation of CXCR4-CXCL12 signalling was responsible for the suppression of migratory capacity of HNSCC cells.

To elucidate the potential mechanism and cellular signalling pathways involved in the regulation in which HPV positive HNSCC manifested in cell migration, further investigation focusing CXCR4-CXCL12 signalling and its interaction with related immune cells is on demand.

5.4 The impact of cisplatin and AMD3100 in cell migration/chemotaxis of HNSCC regarding HPV status

HPV-associated HNSCC were known for a significantly improved 5-year overall-survival and disease-free-survival. (Samuels et al. 2016; Modur, Thomas-Robbins, and Rao 2015; Coordes et al. 2016). Not only that, HPV-associated HNSCC patients presented relatively better therapeutic response rates in induction chemotherapy and radio-chemotherapy in comparison to non-HPV-associated HNSCC patients (Fakhry et al. 2008). Nevertheless, in the comparison between the primary surgical and non-surgical treatment, data did not present a statistical difference as regard to survival in HPV-associated OPSCC patients (Sinha et al. 2018). From a multicenter analysis, HPV 16 status was demonstrated to correlate with loco-regional tumor control in patients with OPSCC (Lohaus et al. 2014).

Cisplatin, as a key cytotoxic anti-cancer agent for advanced HNSCC, presents varying degrees of intrinsic drug resistance(Suzuki et al. 2011). The current therapeutic paradigms have modified in focusing on de-escalation of treatment intensity in order to maximize survival and mitigate treatment-oriented toxicity (Sandulache et al. 2019). Spreafico et al. demonstrated that HPV-associated patients had a survival benefit in the context of the low dose of cisplatin with respect to the non-HPV-associated patients with a survival benefit in high dose of cisplatin (Spreafico et al. 2016). The increased susceptibility of HPV tumors to ionizing radiation and some systemic drugs was attributed to the HPV viral proteins E6 and E7 interfering the regulation of the cell cycle and DNA repair mechanisms (Park et al. 2014; Samuels et al. 2016). Nevertheless, not all HPV-associated OPSCC patients are expected to have favourable outcomes. In vitro chemotherapy, a study pointed out a failure to yield differences in response to platinum-based and other chemotherapeutic agent in HPV positive HNSCC cell lines (Tang et al. 2013).

Recently, the evidence that CXCR4 was involved in the regulation of cisplatin-based chemotherapeutic resistance in tongue squamous cell carcinoma was established (Zhuang and Zhou 2019). Considering the high expression of CXCR4 and CXCL12 in HPV-positive HNSCC cell lines, investigation in our study focused on the impact of treatment from cisplatin and AMD3100 in migration and chemotaxis assay between the HNSCC cell lines relating to HPV status. AMD3100, as CXCR4 antagonist, initially functioned as an agent to blockade infection of $CD4^+$ T cells of human immunodeficiency virus (HIV) (De Clercq 2009). As

collected research demonstrated, AMD3100 was a promising therapeutic agent through the blockage of CXCR4-CXCL12 signalling in inhibiting tumor growth, proliferation and metastasis in vitro and in vivo in several tumor entities (Reeves et al. 2017; Luo et al. 2019; Scotton et al. 2002). In our present study, blockage of CXCR4-CXCL12 by AMD3100 significantly inhibited the cell migration in both of chemotaxis and migration assay in HPV positive HNSCC cells due to its high CXCR4 and CXCL12 expression. Additionally, the prominent suppressed cell motility was observed in HPV positive HNSCC cells in cisplatin and AMD3100 treatment, individually or in combined, in chemotaxis assay of CXCL12 comparing to HPV negative HNSCC cells. Significant therapeutic resistance of cisplatin was not observed in both of HPV negative and positive cell lines. Collectively, these results indicated that the autocrine CXCR4-CXCL12 system in HNSCC cell lines contributed to the cells migration and HPV positive cell lines manifested a more effective susceptibility than negative counterparts in terms of treatment from cisplatin and AMD3100 in chemotaxis assay.

Our results provided an evidence that AMD3100 as an effective therapeutic agent by targeting the CXCR4-CXCL12 axis offered a new option in addition to cisplatin treatment to reach a better loco-regional control while simultaneously having a corresponding cytotoxic effect in HPV-associated HNSCC.

6 Summary

HNSCCs usually exhibit a poor prognosis with regard to locoregional recurrency, distant metastasis and therapeutic resistance. The prevalence of HPV-associated HNSCC dramatically rose in last decade along with a favorable outcome but usually displayed a distant late-metastasis.

Since the CXCR4-CXCL12 axis was demonstrated to take part in tumor cell survival, proliferation, migration and chemotaxis in several solid tumor entities, the prognostic value of CXCR4 and CXCL12 in survival, local control of HNSCC had been confirmed. Recently, the CXCR4-CXCL12 axis was disclosed to play a role in HPV-induced epithelial carcinogenesis.

To investigate the potential character of CXCR4-CXCL12 in HPV-associated HNSCC, the investigation of CXCR4-CXCL12 axis related proteins in primary tumor and further biological behavior experiments in vitro were performed. In our study, with randomly selected OPSCC patients, a significantly high expression of CXCL12 in immunohistochemistry staining was presented in HPV-associated OPSCC primary tumor. In vitro, the high CXCR4 and CXCL12 expression in mRNA in HPV positive HNSCC cell lines were demonstrated through qRT-PCR. Additionally, the significantly high CXCR4 protein expression was proven in immunofluorescence staining in HPV positive HNSCC cell lines. Proliferation assay revealed the involvement of CXCR4-CXCL12 signalling in regulating HNSCC cell proliferation. HPV positive HNSCC cells manifested a more aggressive migratory capability as compared to HPV negative HNSCC cells in migration assay. Nevertheless, in chemotaxis assay with recombinant CXCL12, HPV positive HNSCC cells performed a predominantly suppressed migratory capability. Subsequently in treatment of cisplatin and AMD3100, individually or in combined, AMD3100 exhibited a more effective inhibition in cell motility of HPV positive HNSCC as compared to negative counterpart with a blockage of CXCR4-CXCL12 signalling in migration and chemotaxis assay. Moreover, cisplatin-related drug-resistance was observed neither in HPV positive HNSCC cells nor in HPV negative HNSCC cells.

In consideration of the manifestation of CXCR4-CXCL12 signalling of HPV-associated HNSCC in the progression of tumor cell proliferation and chemotaxis, the understanding of molecular mechanisms considering the interplay of viral integration events, genomic alteration, immune cell subtypes and tumor metabolism are being sought. According to our results, the AMD3100 treatment through blocking CXCR4-CXCL12 signalling to reach

a better loco-regional control and mitigate metastasis risk as regards HPV status in HNSCC might be a promising strategy in future.

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Appendix

AJCC (8th edition) TNM Categories and Definitions for HPV-associated (p16 positive) OPSCC

T categories	**Criteria**
T0	No primary tumor identified
T1	Tumor size ≤2 cm in greatest dimension
T2	Tumor size >2 cm but ≤4 cm in greatest dimension
T3	Tumor size >4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced tumor invading larynx, extrinsic tongue muscles, medial pterygoid, hard palate, or mandible or beyond
Clinical N Category	**Criteria**
Nx	Regional nodes cannot be assessed
N0	No regional nodal metastasis identified
N1	Metastasis to one or more ipsilateral lymph nodes, ≤6 cm
N2	Metastasis to contralateral or bilateral lymph nodes, ≤6 cm
N3	Metastasis in any cervical lymph node >6 cm
Pathologic N Category	**Criteria**
pNx	Regional nodes cannot be assessed
pN0	No regional nodal metastasis identified
pN1	Metastasis to 4 or fewer lymph nodes
pN2	Metastasis to 5 or more lymph nodes
M Category	**Criteria**
M0	Absence of distant metastasis
M1	Presence of distant metastasis

AJCC (8th edition) Prognostic Stage Group for HPV-associated (p16 positive) OPSCC (Clinical)

T categories	N categories	M categories	Stage Group
T0, T1 or T2	N0, N1	M0	I
T0, T1 or T2	N2	M0	II
T3 or T4	N0, N1	M0	II
T3 or T4	N2	M0	III
Any T	Any N	M1	IV

AJCC (8th edition) Prognostic Stage Group for HPV-associated (p16 positive) OPSCC (Pathologic)

T categories	N categories	M categories	Stage Group
T0, T1, or T2	N0, N1	M0	I
T0, T1, or T2	N2	M0	II
T3	N0, N1, or N2	M0	II
T0, T1, T2, T3, or T4	N3	M0	III
T4	N0, N1, N2, or N3	M0	III
Any T	Any N	M1	IV

AJCC (8th edition) TNM Categories and Definitions for non-HPV-associated (p16 negative) OPSCC

T categories	**Criteria**
Tx	Primary tumor cannot be assessed
Tis	Carcinoma in situ
T1	Tumor size ≤2 cm in greatest dimension
T2	Tumor size >2 cm but ≤4 cm in greatest dimension
T3	Tumor size >4 cm in greatest dimension or extension to lingual surface of epiglottis
T4	Moderately advanced or very advanced local disease
T4a	Moderately advanced tumor invading larynx, extrinsic tongue muscles, medial pterygoid, hard palate, or mandible
T4b	Very advanced local disease Tumor invades the lateral pterygoid muscle, pterygoid plates, lateral nasophaynx, or skull base or encases carotid artery
Clinical N Category	**Criteria**
Nx	Regional nodes cannot be assessed
N0	No regional nodal metastasis identified
N1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension and Extranodal Extension (ENE) (–)
N2	Metastasis in a single ipsilateral lymph node, >3 cm but ≤6 cm in greatest dimension and ENE (–); or metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension and ENE (–); or in bilateral or contralateral lymph nodes, ≤6 cm in greatest dimension and ENE (–)
N2a	Metastasis in a single ipsilateral lymph node, >3 cm but ≤6 cm in greatest dimension and ENE (–)
N2b	Metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension and ENE (–)
N2c	Metastases in bilateral or contralateral lymph nodes, ≤6 cm in greatest dimension and ENE (–)

N3	Metastasis in a lymph node >6 cm in greatest dimension and ENE (–); or metastasis in any node(s) and clinically overt ENE (+)
N3a	Metastasis in a lymph node >6 cm in greatest dimension and ENE (–);
N3b	Metastasis in any node(s) and clinically overt ENE (+)
Pathologic N Category	**Criteria**
pNx	Regional nodes cannot be assessed
pN1	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension and ENE (–)
pN2	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension and ENE (+); or >3 cm but ≤6 cm in greatest dimension and ENE (–); or metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension and ENE (–); or in bilateral or contralateral lymph node(s), ≤6 cm in greatest dimension and ENE (–)
pN2a	Metastasis in a single ipsilateral lymph node, ≤3 cm in greatest dimension and ENE (+); or >3 cm but 6 cm in greatest dimension and ENE (–)
pN2b	Metastases in multiple ipsilateral lymph nodes, ≤6 cm in greatest dimension and ENE (–)
pN2c	Metastases in bilateral or contralateral lymph nodes, ≤6 cm in greatest dimension and ENE (–)
pN3	Metastasis in a lymph node >6 cm in greatest dimension and ENE (–); or in single ipsilateral node >3 cm in greatest dimension and ENE (+); or multiple ipsilateral, contralateral or bilateral nodes, any with ENE (+); or a single contralateral node of any size and ENE (+)
pN3a	Metastasis in a lymph node >6 cm in greatest dimension and ENE (–);

pN3b	Metastasis in single ipsilateral node >3 cm in greatest dimension and ENE (+); or multiple ipsilateral, contralateral or bilateral nodes, any with ENE (+); or a single contralateral node of any size and ENE (+)
M Category	**Criteria**
M0	Absence of distant metastasis
M1	Presence of distant metastasis

AJCC (8th edition) Prognostic Stage Group for for non-HPV-associated (p16 negative) OPSCC

T categories	**N categories**	**M categories**	**Stage Group**
Tis	N0	M0	0
T1	N0	M0	I
T2	N0	M0	II
T3	N0	M0	III
T1, T2, T3	N1	M0	III
T4a	N0, N1	M0	IVA
T1, T2, T3, T4a	N2	M0	IVA
Any T	N3	M0	IVB
T4b	Any N	M0	IVB
Any T	Any N	M1	IVC

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