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Matrix Metalloproteinases and Toll-Like Receptors in Early-Stage Oral Tongue Squamous Cell Carcinoma
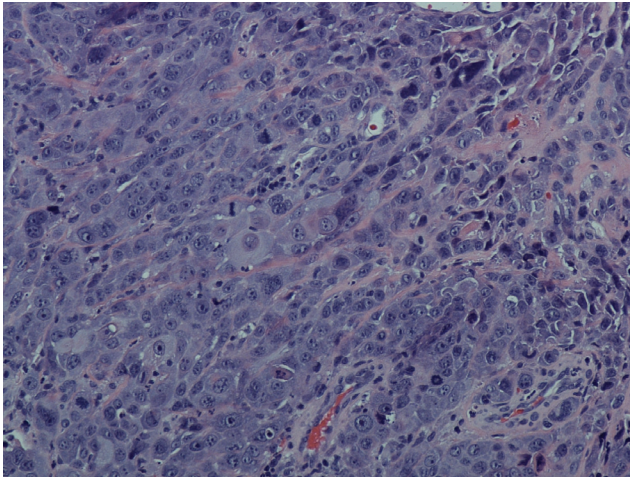
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Matrix metalloproteinases and toll-like receptors in early-stage oral tongue squamous cell carcinoma

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ACADEMIC DISSERTATION

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To René, Venla, Lenni, and Onni

*Doing the best at this moment puts you in the
best place for the next moment.*

Oprah Winfrey

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I   Mäkinen LK, Häyry V, Atula T, Haglund C, Keski-Säntti H, Leivo I, Mäkitie AA, Passador-Santos F, Böckelman C, Salo T, Sorsa T, Hagström J. Prognostic significance of matrix metalloproteinase-2, -8, -9 and -13 in oral tongue cancer. J Oral Pathol Med 2012;41:394-9

II   Mäkinen LK, Häyry V, Hagström J, Sorsa T, Passador-Santos F, Keski-Säntti H, Haukka J, Mäkitie AA, Haglund C, Atula T. Matrix metalloproteinase-7 and matrix metalloproteinase-25 in oral tongue squamous cell carcinoma. Head Neck 2014;36:1783-8

III Mäkinen LK, Atula T, Häyry V, Jouhi L, Datta N, Lehtonen S, Ahmed A, Mäkitie AA, Haglund C, Hagström J. Predictive role of toll-like receptors 2, 4, and 9 in oral tongue squamous cell carcinoma. Oral Oncol 2015;51:96-102

IV Mäkinen LK, Ahmed A, Hagström J, Mäkitie AA, Lehtonen S, Salo T, Haglund C*, Atula T*. Toll-like receptors 2, 4, and 9 in primary, metastasized, and recurrent oral tongue squamous cell carcinomas. Submitted.

*Equal contribution

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ABSTRACT

Predicting the clinical course of an early-stage oral tongue squamous cell carcinoma (OTSCC) is challenging, as even small tumors can behave aggressively. OTSCC often metastasizes to the cervical lymph nodes, and the presence of lymph node metastasis at the time of diagnosis is considered the most important tumor-related prognostic factor in OTSCC. The mechanisms of this disease progression are poorly understood. Despite slight improvement in the prognosis of OTSCC in recent decades, the outcome of these patients is still modest. Therefore, a deeper understanding of the phenomena behind tumor progression would enable medical professionals to evaluate the aggressiveness of the disease and to adjust its treatment more effectively.

The extracellular matrix and basement membrane must be broken down before a tumor can invade surrounding tissues and further spread into blood and lymph vessels. This is a process that involves various proteolytic enzymes, the most important of which are matrix metalloproteinases (MMPs). Over 25 structurally related, but genetically distinct, human MMPs have been identified and characterized: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs. Certain MMPs, especially MMP-8, can also play an anti-metastatic and protective role in tumor development.

Toll-like receptors (TLRs) are pattern-recognition molecules involved in innate immunity that are also expressed in many types of cancer. TLRs apparently play a pivotal role in malignant disease: they are related to tumor progression and, conversely, to cancer inhibition. Their expression pattern and role in oral cancer, however, remains unclear.

In this thesis we studied the expression of MMPs 2, 7, 8, 9, 13, and 25 and TLRs 2, 4, 5, 7, and 9 in early-stage OTSCC, as well as their role in the disease progression and patient outcomes.

The study comprised 73 consecutive clinically T1N0M0 and T2N0M0 OTSCC patients treated at the Helsinki University Hospital, Helsinki, Finland in 1992-2002. We prepared tissue array blocks from primary tumors and immunostained them. We also used whole section slides from patients with metastasized or recurrent disease. We compared immunoexpression of MMPs and TLRs to tumor and patient characteristics as well as to patient outcomes. We also used Western immunoblot to examine TLR-2 and -4 expression in the highly invasive and aggressive HSC-3 OTSCC cell line. In addition, we studied the effect of TLR-2 and -4 antagonist GIT27 (4,5-dihydro-5-isoxasoleacetic acid) on the invasion of the HSC-3 cell line in myoma organotypic invasion assay.

OTSCC tumors expressed both MMPs and TLRs. Nuclear expression of MMP-13, but not cytoplasmic expression of MMP-2, -8, and -9, associated with deeper invasion and advanced tumor size. Furthermore, high nuclear MMP-13 expression predicted poor disease-specific survival. High MMP-7 protein expression associated with the presence of occult cervical metastases, increased invasion depth, and higher tumor grade, and also predicted poor outcome. Immunostaining of MMP-25 failed to correlate with any clinical parameters.

High TLR-2, -4, and -9 expression correlated with deeper tumor invasion. Cytoplasmic expression of TLR-2 and -4 also correlated significantly with higher tumor grade, whereas high TLR-5 expression associated with lower tumor grade. High expression of TLR-9 correlated with advanced tumor size. Negative or mild TLR-5 expression predicted poor disease-specific survival.

OTSCC primary tumors, neck metastases and recurrent tumors expressed TLR-2, -4, and -9. TLR-2 and -4 antagonist GIT27 did not affect the invasion of the HSC-3 cell line in myoma organotypic invasion assay. Thus, TLRs may operate under a different mechanism of action depending on whether they are activated by damage-associated molecular patterns in cancer or by pathogen-associated molecular patterns in infection.

Our results suggest that MMP-7 and MMP-13 in particular may have prognostic value in OTSCC. Their use as prognostic biomarkers, however, calls for further study. TLR-2, -4, and -9 seemed to predict invasive tumor growth. Primary tumors and neck metastases as well as recurrent tumors of OTSCC express these TLRs, suggesting that TLRs seem to play a role in both the development and progression of tongue carcinoma.

TIIVISTELMÄ

Kielisyövän käyttäytymisen ennustaminen on haastavaa, sillä jo pienet kielisyövät saattavat lähettää etäpesäkkeitä taudin varhaisessa vaiheessa. Etäpesäkkeiden esiintymistä kaulan imusolmukkeissa pidetään merkittävimpänä kasvaimeen liittyvänä tekijänä taudin ennustetta arvioitaessa. Huolimatta kielisyövän ennusteen hienoisesta paranemisesta viime vuosikymmenien aikana, taudin elossaoloennuste on edelleen varsin huono ja taudin etenemiseen vaikuttavat mekanismit tunnetaan huonosti. Näistä syistä johtuen olisi toivottavaa tuntea tarkemmin syövän taustalla piileviä biologisia mekanismeja, jotta taudin aggressiivisuutta voitaisiin arvioida paremmin ja mahdollisesti löytää myös kohdennettuja hoitomuotoja.

Jotta kasvainsolut voivat edetä ympäröivään kudokseen sekä veri- ja imusuoniin, proteolyyttisten entsyymien pitää hajottaa soluväliainetta ja tyvikalvo. Matriksin metalloproteinaaseilla (engl. matrix metalloproteinases, MMP) on tässä keskeinen osuus. Nykyisin tunnetaan yli 25 rakenteellisesti toisiaan muistuttavaa, mutta geneettisesti erilaista, MMP:a, jotka jaetaan eri ryhmiin: kollagenaasit, gelatinaasit, stromelysiinit, matrilysiinit, solukalvoihin liittyvät ja muut MMP:t. Aikaisempien tutkimusten valossa, eräät MMP:t, etenkin MMP-8, omaavat toisaalta myös syövältä suojaavia vaikutuksia.

Tollin kaltaiset reseptorit (engl. toll-like receptors, TLR) ovat proteiineja, jotka tunnistavat elimistölle vieraita ja myös elimistön omia rakenteita, ja jotka säätelevät luonnollista immuniteettia. Tiettyjen TLR:ien on todettu ilmentyvän myös eräissä syövissä. TLR:eilla on havaittu sekä syöpää edistäviä että hidastavia vaikutuksia, mutta niiden merkitys, ja osin myös ilmentyminen, suusyövässä on vielä epäselvä.

Tässä väitöskirjatyössä tutkittiin MMP:ien 2, 7, 8, 9, 13 ja 25:n sekä TLR:ien 2, 4, 5, 7 ja 9 ilmentymistä varhaisvaiheen kielisyövissä sekä niiden merkitystä kasvaimen etenemisessä ja taudin ennusteessa.

Tutkimusmateriaalimme koostui 73 potilaasta, joilla oli todettu kliinisesti T1N0M0 tai T2N0M0 kielisyöpä. Potilaat oli hoidettu Helsingin yliopistollisessa keskussairaalassa vuosina 1992-2002. Emokasvaimista tehtiin tissue array -blokit, jotka värjättiin immunohistokemiallisin menetelmin. Kasvaimet, jotka olivat uusiutuneet tai lähettäneet etäpesäkkeitä, tutkittiin myös kokoleikkeitä käyttäen. Tutkittujen MMP:ien ja TLR:ien ilmentymistä syöpäkudoksessa verrattiin kasvaimen muihin ominaisuuksiin, potilaaseen liittyviin tekijöihin sekä taudin ennusteeseen. TLR-2:n ja -4:n ilmentymistä tutkittiin myös erittäin invasiivisessa ja aggressiivisessa HSC-3 kielisyöpäsolulinjassa. Lisäksi tutkimme TLR-2:n ja -4:n vastavaikuttaja GIT27:n (4,5-dihydro-5-isoxasoleacetic acid) vaikutusta kielisyöpäsolujen invaasioon myoomamallissa.

MMP:t ja TLR:t ilmentyivät suuressa osassa kielisyöpäkasvaimia. MMP-13:n lisääntynyt ilmentyminen tumissa liittyi kasvaimen syvempään invaasioon ja suurempaan kokoon sekä ennusti potilaiden lyhyempää elossaoloaikaa. Sen sijaan samanlaista vaikutusta ei todettu MMP-2:n, -8:n ja -9:n ilmentymisellä syöpäsolujen solulimassa. MMP-7 ilmentyi voimakkaammin niillä potilailla, joilla todettiin kaulan etäpesäkkeet, kasvaimen syvempi invaasio tai huonompi erilaistumisaste. Lisääntynyt MMP-7-ekspressio ennusti myös potilaiden lyhyempää elossaoloaikaa. MMP-25:n ilmentyminen ei liittynyt mihinkään tutkittuun kliiniseen tekijään.

Voimakas TLR-2, -4 ja -9 ilmentyminen liittyi kasvaimen syvempään invaasioon. Soluliman vahva TLR-2 ja -4 ilmentyminen liittyi lisäksi kasvaimen huonompaan erilaistumisasteeseen, kun taas TLR-5–proteiinia esiintyi enemmän korkeasti erilaistuneissa kasvaimissa. TLR-9:n ilmentyminen oli voimakkaampaa kookkaissa kasvaimissa. Puuttuva tai vähäinen TLR-

5:n ilmentyminen puolestaan ennusti lyhyempää tautikohtaista elossaoloaikaa. TLR-2, -4 ja -9 ilmentyivät sekä kielisyövän emokasvaimissa että kaulan etäpesäkkeissä ja uusiutuneissa kasvaimissa. TLR-2:n ja -4:n vastavaikuttaja GIT27 ei vaikuttanut kielisyöpäsolujen invaasioon myoomamallissa. TLR:iden vaikutusmekanismi on mahdollisesti erilainen riippuen siitä, aktivoituvatko ne syövästä vai tulehduksesta johtuen.

Tutkimuksemme perusteella etenkin MMP-7:lla ja -13:lla näyttäisi olevan ennusteellista merkitystä kielisyövässä. TLR-2, -4 ja -9 proteiinien ilmentyminen säätelee mahdollisesti kielisyövän invaasiota. Nämä TLR:t ilmentyivät sekä emokasvaimissa, kaulan etäpesäkkeissä että uusiutuneissa kasvaimissa, joten on todennäköistä, että TLR:eilla on jonkinlainen rooli niin kielisyövän synnyssä kuin sen etenemisessäkin.

ABBREVIATIONS

cfDNA	Cell-free DNA
cN0	No clinically evident nodal metastases
cN+	Clinically evident nodal metastases
CRT	Chemoradiotherapy
CSCC	Cutaneous squamous cell carcinoma
CT	Computed tomography
ctDNA	Circulating tumor DNA
DAB	3,3-diaminobenzidine tatrahydrochloride
DAMP	Damage-associated molecular pattern
DFS	Disease-free survival
DSS	Disease-specific survival
ECM	Extracellular matrix
ECS	Extracapsular spread
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
END	Elective neck dissection
GIT27	4,5-dihydro-5-isoxasoleacetic acid
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSC-3	Human squamous cell carcinoma cell line
IHC	Immunohistochemistry
kDa	Kilodalton
LP	Laryngeal papillomatosis
LPS	Lipopolysaccharide
LSCC	Laryngeal squamous cell carcinoma
LTA	Lipoteichoic acid
MAP	Mitogen-associated protein
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MT-MMP	Membrane-type matrix metalloproteinase
MyD88	Myeloid differentiation primary response gene 88
ND	Neck dissection
NF-κB	Nuclear factor kappa beta
NGS	Next-generation sequencing
NPC	Nasopharygeal carcinoma
OPC	Oropharyngeal cancer
OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
OSCC	Oral squamous cell carcinoma
OTSCC	Oral tongue squamous cell carcinoma
PAMP	Pathogen-associated molecular pattern
PET-CT	Positron emission tomography–computed tomography
pN+	Pathologically positive nodal metastases
Poly(I:C)	Lyophilized polyinosinic-polycytidylic acid
PRR	Pattern-recognition receptor
RT	Radiotherapy
SCC	Squamous cell carcinoma
SNB	Sentinel lymph node biopsy
TIMP	Tissue inhibitor of metalloproteinase
TLR	Toll-like receptor
TMA	Tissue microarray
TNM	Tumor node metastasis

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, and half of these malignancies occur in the oral cavity (1). In Finland, approximately 3% of newly diagnosed cancers appear in the head and neck region (2).

Oral tongue squamous cell carcinoma (OTSCC) is the most common cancer in the oral cavity. In Finland, 128 new cases of OTSCC were diagnosed in 2012, with male gender dominating slightly (2). The incidence of OTSCC shows significant geographic variation worldwide; incidence is stable or falling in some regions, whereas in other areas, such as in Finland, incidence has risen slighly in recent decades (3-6).

The well-known risk factors for OTSCC include tobacco use and alcohol consumption, and their carcinogenic effects are multiplicative when combined (7-9). In addition, research suggests that poor oral hygiene and dietary factors contribute to oral carcinogenesis (10). A portion of oral premalignant lesions; leukoplakia, erythroplakia, and lichen planus, also progress to invasive carcinomas (7). Research has also shown an association between human papilloma virus (HPV) and oral squamous cell carcinoma (OSCC) (11). However, the precise role of HPV in OTSCC and the value of its surrogate marker, p16, remain inconclusive.

Patients with OTSCC often present with tongue ulcers, throat pain, or a painless neck lump. Diagnosis is based on the clinical examination and histological assessment of a tumor biopsy. OTSCC spreads primarily through the lymphatic vessels and thus typically reaches the lymph nodes on the neck first. Evaluating the primary tumor and regional metastasis generally requires computed tomography (CT) or magnetic resonance imaging (MRI) or both. CT of the chest also serves to rule out second primary tumors, which are relatively common, as well as distant metastases in cases of advanced diseases (12-15).

The primary treatment of OTSCC is generally based on surgery, including resection of the primary tumor and neck dissection when indicated (16,17). Patients who present with advanced tumor size, a deeply invasive primary tumor or positive lymph nodes receive postoperative radiation or chemoirradiation treatment (18).

OTSCC is a disease with a modest survival rate; approximately one third of patients die from the disease, and the five-year disease-specific survival (DSS) for advanced-stage (III-IV) patients is only 30 to 50% (19-24). OTSCC reportedly has a higher rate of metastases and exhibits more aggressive behavior than do other carcinomas of the oral cavity, and even small tumors may metastasize in the early course of the disease (23,25-27). The presence of lymph node metastasis is considered the most important tumor-related prognostic factor in OTSCC (28-31). In addition to the clinical stage of the disease, which includes primary tumor size and invasion, neck node involvement and the possible presence of distant metastasis, the invasion pattern, perineural invasion, the carcinoma's proximity to resection margins, the presence of extracapsular spread (ECS), and histopathological grade all have prognostic significance in OTSCC (20,25,27,29,30,32-42).

The extracellular matrix (ECM) and the basement membrane (BM) must be broken down before the tumor can spread into the blood and lymph vessels and, thus, metastasize, which crucially involves, among other proteolytic enzymes, matrix metalloproteinases (MMPs). Additionally, MMPs process several non-matrix bioactive substrates, such as cytokines, chemokines, immune mediators, other proteases, and cell adhesion proteins, which, consequently, direct immune responses (43). Over 25 human MMPs have been identified,

characterized, and divided into six groups according to their structure and substrate specificity: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs (44-47). MMPs are connected to invasion and metastasis in cancer (48), but are also shown to play an anti-metastatic and protective role in tumor development (48-52). Studies have found that overexpression of MMP-1, -2, -3, -7, -9, -13 and -14 in HNSCC contributes to invasion and metastasis and predicts poor survival (53-59). In contrast, MMP-8 overexpression in OTSCC has associated with higher survival rates (51).

Toll-like receptors (TLRs), pattern-recognition proteins involved in innate and adaptive immunity, have both endogenous and exogenous ligands. Ten human TLRs have been identified, each of which recognizes a specific microbial component (60). Many types of cancer also express TLRs, which are related to both tumor progression and cancer inhibition (61-64). Studies have shown that TLRs are expressed in the HNSCCs of different subsites (65-77). However, their precise expression pattern and role in HNSCC remains unclear.

A deeper understanding of the metastatic potential and the aggressiveness of the disease would be useful. Given the fairly poor survival outcome of these patients, as well as the considerable morbidity resulting from treatment, more individually tailored treatment based on more thorough knowledge of tumor behavior is necessary. Biomarkers could supply such information. This study aims to investigate the role of MMPs and TLRs in OTSCC.

1 REVIEW OF THE LITERATURE

1.1 Oral tongue squamous cell carcinoma

1.1.1 Epidemiology and etiology

The oral cavity is a common site for squamous cell cancers of the upper aerodigestive tract, probably because it is the first entry point for many carcinogens (78). Globally, 5% of cancers occur in the head and neck region, and approximately half of those occur in the oral cavity (1). The prevalence and incidence of oral cancer varies widely across countries (4,5). In Finland, head and neck cancer (HNC) comprises approximately 3% of all diagnosed cancers, oral cancer being the most common subtype (2). The Finnish Cancer Registry (www.cancerregistry.fi) reports that 2012 saw 128 newly diagnosed cases of OTSCC, with men representing 59% of cases (2). In recent decades, the incidence of OTSCC in Finland has been rising modestly (3,6). Patients diagnosed with OTSCC are often younger and healthier than patients diagnosed with oral cancer at other subsites (79).

Over 90% of oral cavity cancers are squamous cell carcinomas. Known risk factors include tobacco in different forms and alcohol, which together account for about 75% of HNCs; their effects are multiplicative when combined (5,7-9). Some evidence also reveals the influence of poor oral hygiene and dietary factors on the development of HNC (10). Most oral squamous cell carcinomas (OSCC) arise from premalignant lesions, such as leukoplakia, erythroplakia, and lichen planus. However, predicting which lesions will progress to invasive carcinoma is challenging (7). Studies have identified HPV as a significant contributor to oropharyngeal cancer (OPC) as well as its association with improved response to traditional treatment, whereas the incidence of HPV-related oral cancer is relatively low, and the influence of HPV on OSCC is currently unclear (80-82). However, a recent meta-analysis by Syrjänen et al. showed a strong association between oral potentially malignant disorders and HPV and OSCC (11).

Genetic predisposition also plays a role in the development of HNC; studies have linked a family history of HNC in a first-degree relative as well as genetic polymorphisms in genes encoding enzymes involved in the metabolism of tobacco and alcohol to an elevated risk for developing the disease (5,83).

1.1.2 Diagnosis and classification

The diagnosis of OTSCC is based on clinical and histological examination of a tumor biopsy. Evaluating the size, extent and infiltration of the primary tumor, as well as the presence of regional metastases, generally requires MRI or CT. Evaluation of the neck can also require ultrasound and ultrasound-guided fine-needle aspiration cytology. CT of the chest is widely used to evaluate distant metastasis and second primary tumors. Selective cases (e.g., when an uncertain radiological finding requires further evaluation) may require positron emission tomography-computed tomorgaphy (PET-CT) (12-15).

However, not all imaging can successfully identify micrometastases. The detection of micrometastasis may require a sentinel lymph node biopsy (SNB) (84,85). Some centers favor SNB: in a case of cT1 tumors with cN0 neck, for example, SNB, with a sensitivity of over 90%, is recommended (84,86).

Precise staging (Table 1) (87), based on clinical examination and radiological assessement, guides therapeutic decisions. Even though staging of the disease is the basis for treatment planning, this practice fails to take into account pathological or

biological parameters other than tumor size, location, invasion and the presence of metastasis such as infiltration depth, grade or immunohistological markers (8).

Table 1. TNM and stage classification for oral cavity squamous cell carcinoma.

T – Primary tumor

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	≤ 2 cm
T2	> 2 cm to 4 cm
T3	> 4 cm
T4a	Invades through cortical bone into deep/extrinsic muscles of the tongue, maxillary sinus, skin
T4b	Invades masticator space, pteygoid plates, skull base, encases the internal carotid artery

N – Regional lymph nodes

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node ≤ 3 cm
N2	N2a Metastasis in a single ipsilateral lymph node > 3 cm to 6 cm
	N2b Metastasis in multiple ipsilateral lymph nodes ≤ 6 cm
	N2c Metastasis in bilateral or contralateral lymph nodes ≤ 6 cm
N3	Metastasis in a lymph node > 6 cm

M – Distant metastasis

M0	No distant metastasis
M1	Distant metastasis

Stage grouping

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

Modified from TNM classification of malignant tumours, 7th edition, Sobin L.H., Gospodarowicz M.K., Wittekind Ch, editors. Wiley-Blackwell, New York, 2009 (87).

1.1.3 Treatment

Treatment of the primary tumor

Treatment planning is based on general guidelines, which can vary across centers and countries. Surgery alone is considered adequate in cases of early-stage disease (i.e., small local tumors) (36). Larger tumors, tumors with deep infiltration, and those with metastases are generally treated with a combination of surgery and postoperative radiation or chemoirradiation (21,88). Surgical treatment includes resection of the primary tumor and neck dissection when indicated. Larger tumors often require reconstruction (88). OSCCs may be less sensitive to radiation and chemotherapy than are oropharyngeal or laryngeal cancers (78).

Treatment of the neck

The lymph nodes of the neck are generally treated electively even though they are clinically normal, but if the risk for occult metastasis exceeds 15 to 20%. When the risk is lower, one can adopt a wait-and-see policy or perform a sentinel lymph node biopsy (7,30,89-91). Such elective treatment is generally surgical: elective neck dissection (END), often to neck levels ranging from I to IV (16,17). In practice, large T1 or more advanced tumors or tumors with deep invasion demand elective neck treatment. Abnormal lymph nodes detected during clinical examination usually undergo extended therapeutic neck dissection. Patients with histopathologically confirmed neck metastasis receive postoperative radiation or chemoirradiation treatment.

Oncological treatment

Advanced tumor size, deeply invasive primary tumor or positive lymph nodes call for postoperative radiotherapy to improve local disease control (8,78). In addition, positive surgical margins require further treatment, either re-operation or radiotherapy or both. Conventional radiation comprises 50 Gy for the neck and 56-70 Gy for the primary tumor area. Postoperative chemoradiotherapy, most often with cisplatin, is administered when multiple lymph nodes are involved or present extracapsular spread (ECS) or both (18). Chemotherapy may prolong survival by 8 to 22% (92).

1.1.4 Prognosis and prognostic factors

Even though the prognosis of OTSCC has improved slightly in recent decades, OTSCC remains a disease of modest survival, with approximately one third dying of the disease (19,21,22). In early-stage OTSCC, the five-year DSS is reportedly around 60 to 80%, whereas in advanced stages of the disease, the five-year DSS drops to 30 to 50% (20-24). A multicenter international study comparing the outcomes of 2738 patients treated for OSCC between 1990 and 2000 to those between 2001 and 2011 showed a significant improvement from 59 to 70% in the OS of these patients (93). In Finland, the incidence of OTSCC has been rising in recent decades, yet reports indicate a slight improvement in survival: the five-year age-standardized relative survival rose from 43 to 50% among men and from 55 to 67% among women during 1964-2003 (6). Approximately two-thirds of HNSCC patients present with advanced-stage disease commonly involving regional lymph nodes, and about 10% of patients present with distant metastasis at the time of diagnosis (8). Reports indicate that OTSCC has a higher metastasis rate and behaves more aggressively than other carcinomas of the oral cavity (25-27,39), but controversial results have shown no differences in survival between OTSCC and OSCCs of other subsites (94). The presence of occult neck metastases in early-stage

OTSCC is relatively high and predicts survival outcome (23,35). Furthermore, despite aggressive therapy, patients with stage I-II OTSCC often present with a lower rate of local tumor control and poorer survival than with many other types of cancer at an early stage (95,96).

Patient-related prognostic factors

Sociodemographic factors are considered to have only weak prognostic value in OSCC (34). Furthermore, age and gender as prognostic parameters in OTSCC remain controversial (26,97,98). Among patients with stage III–IV carcinomas, heavy alcohol consumption has significantly associated with poor disease-specific survival (99). High alcohol consumption has also associated with higher rates of recurrent disease and second primary tumors (100).

Clinocopathological prognostic factors

TNM (tumor, node, metastasis) stage is the most important tumor-related prognostic factor in HNC, and the presence of neck metastases reduces the five-year survival rate by 50% (25,29,30,32,33,35,36,39). Other clinicopathological factors of prognostic signifigance in OTSCC include the pattern of invasion, perineural invasion, the proximity of the carcinoma to resection margins, the presence of ECS, and histopathological grade (20,27,34,37,38,40-42).

In a study of 2258 OSCC patients with pathologically negative neck nodes, clinical nodal stage proved to be an independent prognostic predictor of outcome (101). Sayed et al. proposed that the lymph node ratio (the ratio of total positive nodes to total dissected nodes) would be a more accurate prognostic marker than the current N staging of TNM classification in OSCC (102). A recent study of 1617 OSCC patients showed that tumor size, nodal status, tumor subsite and bone invasion

were the most influential predictors of disease recurrence and cancer-specific mortality probability (103).

Research has shown that the ECS of cervical lymph node metastasis decreases the five-year survival rate by more than 50%, and ECS is considered a particularly reliable predictor for disease recurrence and death from HNSCC (25,29,104).

The best histological predictor of local metastasis and poor survival may be the infiltration depth of the tumor (20,27,34,40,41). Tai et al. found that increased tumor thickness with perineural invasion predicts lower disease-specific survival and a higher lymph node metastasis rate in OTSCC and buccal SCC (38). In addition, multifocal perineural invasion alone has associated with with worse prognosis in OSCC (42). Moreover, infiltration depth has proved to be one of the most important prognostic factors in OSCC, and some researchers have proposed including it in the TNM staging system (39). However, the value of tumor infiltration for optimal treatment planning is limited due to poor specificity in identifying high-risk patients, and the optimal cut-off for tumor thickness to define high-risk categories remains uncertain (35,39). Additionally, techniques for measuring the depth of infiltration (or tumor thickness) have differed substantially between studies, so the results are difficult to compare. Infiltration depth can be measured either from the surface of the tumor to the deepest point of invasion or from the level of the adjacent mucosal lining.

Several researcers have pointed out the significance of resection margins in OSCC. Research has shown that compromised resection margins predict local recurrence and poor survival (20,105,106). However, what exactly constitutes a safe margin remains controversial (107-109).

In OSCC, evidence suggests that, regardless of the size of the primary tumor,

patients with poorly differentiated tumors show a higher rate of local metastasis at presentation than do patients with well differentiated tumors (31,110). In a large population-based analysis of early-stage OSCC, high histologic grade associated with poor survival and carried independent prognostic value (111). However, the subjective nature of tumor grade evaluation and its poor specificity limit its prognostic value (27,31,35,94). In a multivariate analysis of T1-2N0-1 tongue carcinoma patients, Shim et al. reported an association of higher tumor grade and deeper invasion with poor DSS, whereas advanced T classification failed to predict prognosis (37). Bonnardot et al. made the same finding in early-stage (T1-T2) OTSCC; poor histological differentiation and tumor thickness increased risk of death (22).

Tumor budding is a specific type of invasive growth in carcinomas characterized by single invading tumor cells or small clusters of tumor cells (< 5 cells) at the invasive front of the tumor. These cells are more invasive than cells in the main tumor mass. A recent review of tumor budding in HNSCC suggested a strong association between tumor budding and tumor progression, as well as a strong correlation with prognosis (112). Furthermore, a retrospective multicentre study of tumor budding in early-stage OTSCC found a correlation between high tumor budding and DSS (113). Another study by the same group introduced a novel prognostic model for early-stage OTSCC which combined tumor budding and depth of infiltration into a BD-model (114). Multivariate analysis showed that a high-risk BD-score correlated significantly with loco-regional recurrence and DSS. Xie et al. also evaluated tumor budding in early-stage OTSCC and found that tumor budding independently predicted the prognosis of these patients (115). However, before adapting tumor budding in daily practice to evaluate histological HNSCC specimens,

standardization of scoring methods and the risk stratification cut-off point is essential.

Tumorigenesis, tumor microenvironment and molecular markers in HNSCC

Hanahan and Weinberg have identified eight hallmarks of cancer to describe the multistep and complex process of tumorigenesis (116). These hallmarks include the ability of cancer cells to sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, activate invasion and metastasis, reprogram energy metabolism and evade immune destruction. In addition, underlying these hallmarks are genome instability, a tumor microenvironment consisting of normal cells interacting with cancer cells, and inflammation, all of which contribute to these hallmark functions. MMPs contribute to tumor angiogenesis and cancer cell invasion and metastasis, whereas TLRs are involved in inflammation and cancer cells evading immune destruction.

Research has shown the tumor microenvironment to be a dynamic state containing a variety of components, including non-cancerous cells (i.e., immune cells, fibroblasts, angiogenic vascular cells) and an extra-cellular matrix milieu (consisting of fibers, mainly collagen and fibronectin, and soluble factors such as enzymes, growth factors, cytokines and chemokines) interacting with cancer cells (117,118). Therefore, modifications in these components of the tumor microenvironment should influence the growth, invasion and spread of cancer cells. Currently, no therapy targeting the tumor microenvironment in OSCC is in clinical use (118).

Epithelial-mesenchymal transition (EMT) plays a crucial role in cancer progression. Cancer cells undergoing EMT

can acquire invasive properties and further enter to stroma, resulting in a favourable microenvironment for cancer progression and metastasis. Han et al. investigated EMT through the measurement of E-cadherin (a transmembrane protein, which plays an important role in cell adhesion forming adherent junctions to bind cells within tissues together) and vimentin (a protein that is expressed in mesenchymal cells) in different stages of OTSCC (119). They found that EMT expression correlated with lymph node metastasis and DFS.

Several molecular markers have been studied in HNSCC recently, with some of these markers also being adapted to clinical practice. MIB-1, for example, is widely used in evaluating salivary gland carcinomas (120,121). p16, a surrogate marker for HPV, is currently in clinical use in the diagnostics of OPC (122,123). Epidermal growth factor receptor (EGFR) has been widely examined in HNSCC (124), and cetuximab, an anti-EGFR 1 monoclonal antibody, is commercially available and has been used to treat locally advanced HNC in combination with radiation therapy (18). Additionally, in OTSCC, several molecular markers have been studied, including oncoprotein cancerous inhibitor of PP2A (CIP2A) (125), Bmi-1, a protein that controls the cell cycle and the self-renewal of tissue stem cells (126), hypoxia-inducible factor-1α (HIF-1α) and cyclooxygenase-2 (COX-2) (127), as well as numerous other proteins (27).

The role of biomarkers in decisions about the treatment of cN0 neck in HNSCC was evaluated in a review by Takes et al., who concluded that biomarkers may provide additional and complementary information and may even be able to identify patients at low risk for occult metastasis, but that due to the complexity of the metastatic process, identifying a single marker for metastasis is unlikely (128). Instead, techniques enabling the study of many factors simultaneously look promising.

Wikner et al. have reviewed circulating tumor cells in OSCC and suggest that OSCC is a systemic disease and that the investigation of circulating tumor cells from peripheral blood samples could offer new perpectives on the disease and provide an opportunity to identify potential targets for individualized therapies (129). Furthermore, the assessment of circulating tumor cells could aid in predicting disease recurrence.

Cell-free DNA (cfDNA), meaning all the DNA outside the cells, is currently considered one of the most promising tumor markers (130-132). Circulating tumor DNA (ctDNA) is cell-free DNA released from tumor cells. ctDNA is present in a patient's plasma and can serve, for example, in primary cancer diagnostics, the evaluation of treatment response and resistance, and the detection of residual disease. Next-generation sequencing (NGS), a technique that can identify rare mutant variants in complex mixtures of DNA, can serve in the further analysis of ctDNA (130).

1.2 Matrix metalloproteinases

1.2.1 Classification, expression, and regulation of MMPs

MMPs are enzymes that can degrade almost all ECM and BM proteins, which is essential for tumor cells to spread into the blood and lymph vessels and adjacent tissues (133). BM is a continuous sheet that supports cell layers, such as the epithelium and endothelium, and ECM is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells (133,134). ECM consists of structural proteins such as collagen and elastin; specialized proteins such as fibrillin, fibronectin, and laminin; and proteoglycans. Most carcinomas

Table 2. MMPs, their common names and main substrates.

MMP subclass		Common name	Main substrates
Collagenases	MMP-1	Collagenase-1	Collagen (I-III, VII, X, XI), gelatin, casein, perlecan, entactin, aggregan, tenascin, laminin, proMMP-1, -2, and -9
	MMP-8	Collagenase-2	Collagen (I-III, VII, X), gelatin, entactin, aggrecan, tenascin, proMMP-8
	MMP-13	Collagenase-3	Collagen (I-IV, VII, IX, XV, XVIII), gelatin, entactin, tenascin, aggrecan
Gelatinases	MMP-2	Gelatinase-A	Gelatin, collagen (I, III, IV, V, VII, X, XI), elastin, fibrinogen, plasminogen, laminin, aggregan, vitronectin, decorin
	MMP-9	Gelatinase-B	Gelatin, collagen (I, IV, V, VII, X, XI, XVIII), elastin, vitronectin, fibronectin, laminin, proMMP-2 and -9
Stromelysins	MMP-3	Stromelysin-1	Aggregan, fibronectin, laminin, gelatin, collagen (III, IV, V, IX, X, XI, XVIII)
	MMP-10	Stromelysin-2	Collagen (I, III, IV), gelatin, elastin, proMMP-1, -8, and -10
	MMP-11	Stromelysin-3	Fibronectin, gelatin, laminin, aggregan
Matrilysins	MMP-7	Matrilysin-1	Fibronectin, gelatin, laminin, aggregan, collagen (I, IV, V, IX, X, XI, XVIII), Fas ligand
	MMP-26	Matrilysin-2	Collagen (IV), gelatin, proMMP-9
MT-MMPs	MMP-14	MT1-MMP	Collagen (I-III), gelatin, aggregan, laminin, proMMP-2 and -13
	MMP-15	MT2-MMP	Proteoglycans, proMMP-2
	MMP-16	MT3-MMP	Collagen (III), fibronectin, proMMP-2
	MMP-17	MT4-MMP	Gelatin, fibrinogen, proMMP-2
	MMP-24	MT5-MMP	Fibronectin, gelatin, proMMP-2
	MMP-25	MT6-MMP	Collagen (IV), gelatin, proMMP-2 and -9
Other MMPs	MMP-12	Macrophage elastase, metalloelastase	Collagen (I, IV), elastin, fibronectin, laminin, proteglycans, fibrinogen
	MMP-19	MMP RASI-1	Collagen (I, IV), laminin, gelatin, tenascin
	MMP-20	Enamelysin	Amelogenin, aggregan, laminin
	MMP-21	-	Gelatin
	MMP-23	Cysteine array (CA-) MMP	Gelatin
	MMP-27	C-MMP	Not known
	MMP-28	Epilysin	Casein

Modified from Johansson et al. 2000, Wahlgren et al. 2001, Chakraborti et al. 2003, Kessenbrock et al. 2010, and Hua et al. 2011 (44-47,133,137).

overexpress these ECM proteins. In addition, MMPs process several non-matrix bioactive substrates, such as cytokines, chemokines, immune mediators, other proteases, and cell adhesion proteins, thereby modifying various cellular, immunologic, intracellular, apoptotic and anti-inflammatory responses (135,136). Thus, MMPs play key roles in inflammation and carcinogenesis, and generally in maintaining ECM homeostasis.

Researchers have identified and characterized over 25 different MMPs with unique structures and diverse functions: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and other MMPs (Table 2) (44-47,133,137).

In intact normal tissues, the activity and expression of most MMPs are maintained at undetectably low or quiescent levels (43). Since MMPs can degrade almost all tissue and structural components of connective tissue matrix and BMs, their activities require regulation at multiple levels: transcription, secretion, inhibition of activity, and changes in MMP localization either inside or outside the cell (47). Several pro-inflammatory cytokines, hormones and growth factors can activate MMP gene transcription. Activation of inactive proenzymes (proMMPs) can occur in different localizations: intracellularly, at the cell surface by MT-MMPs, or extracellularly by other proteases (138). MMPs can be activated by, for example, plasmin, trypsin, cysteine proteinases, and bacterial and candidal proteinases. Activated MMPs can further participate in the processing and activation of other MMPs in mutual activation cascades (43). Evidence suggests that the same MMP can have opposite effects based on the cell type in which expression occurs (49).

The function of different MMPs depends on the local balance between them and their physiological inhibitors. Tissue-inhibitors of matrix metalloproteinases (TIMP) are endogenous inhibitors of MMPs consisting of four members (TIMPs 1-4). TIMPs share some structural features, but also exert specific and selective distinct biochemical characteristics and expression profiles (133). TIMPs 1-4 can inhibit all MMPs, but TIMP-1 is a relatively ineffective inhibitor of MMP-19 and MT-MMPs (43). The primary function of TIMPs is considered MMP inhibition, but they also serve other functions such as MMP transportation and stabilization, MMP localization to the cell surface via MT-MMP binding, inhibition of angiogenesis, promotion of bone-resorbing activity, and growth factor-like activity, as well as pro- and anti-apoptotic functions (134).

Researchers have studied the expression and activity of different MMPs in a variety of human cancers. MMP activation is linked to tumor progression both in early tumorigenesis (e.g., in malignant transformation, angiogenesis and tumor growth) and in late-stage tumor progression (48,133). Most prognostic studies have linked elevated levels of different MMPs to tumor progression, invasion, metastasis and poor survival, but other researchers have also described an association with improved survival (49,51,139). Studies have shown that MMP-8, for example, has a protective influence against the spread of cancer in skin (52), breast (140), and tongue (51) cancers.

Studies have also reported upregulated production and enhanced MMP activation in breast, gastric, lung, vulva, and head and neck cancers, chondrosarcomas, and malignant melanomas (44,139), Furthermore, studies have shown that MMP overexpression in these cancers predicts tumor recurrence, metastasis, and invasion (44,139). Increased production of various MMPs (MMP-1, -2, -3, -7, -9, -13, and -14) reportedly associates with poor tumor differentiation, distant metastases, and shorter survival (48).

Some preliminary studies have focused on MMP inhibitors and cancer treatment also. Up-regulation of TIMPs in tumor cells may inhibit tumor invasion and metastasis, but TIMPs also serve functions not performed by synthetic MMP inhibitors (141). Although preclinical studies of the efficacy of MMP inhibitors in tumor models have been encouraging, the results of clinical trials in cancer patients have been somewhat disappointing, leading in some cases to tumor progression or severe side-effects (49,133). Furthermore, since most of the clinical trials have been conducted in patients with late-stage disease, the question of whether MMP inhibitors benefit early-stage cancer treatment remains unanswered (133).

1.2.2   Collagenases

Collagenases (MMP-1, -8, and -13) regulate cell growth and survival, and are thus strongly linked to cancer development and the invasion capacity of tumors as well as to various inflammatory conditions (45). Collagenases differ in their activation, substrate specificities, functional roles, and cellular localization (44).

MMP-8 has a wide substrate specificity, and its activities overlap with those of other collagenases (142). MMP-8 may also play an anti-metastatic and protective role in tumor development as well as exhibit anti-inflammatory characteristics by processing anti-inflammatory cytokines and chemokines and regulating inflammatory cell apoptosis and immune response (48-52,142,143).

MMP-13, a catalytically potent enzyme, seems to have a limited expression, but broad substrate specificity (142). Overexpression of MMP-13 occurs in many conditions of pathological tissue destruction, such as inflammation, infection and various types of cancer of invasive capacity (43).

1.2.3   Gelatinases

Gelatinases, MMP-2 and -9, have rather similar substrate specificity cleave different types of collagen and gelatin, and are, among other proteins, linked primarily to angiogenesis (44,133,135). However, the role of MMPs in angiogenesis seems uncertain, and evidence suggests that the effects of MMPs in angiogenesis may be context-dependent (133,135). Studies have shown that various cell lines such as keratinocytes, osteoclasts, eosinophils, neutrophils and macrophages express MMP-9, and that many inflammatory cytokines can activate it (43). Thus, MMP-9 overproduction and activation occurs in several inflammatory and malignant diseases.

1.2.4   Matrilysins

MMP-7 (matrilysin-1) and MMP-26 (matrilysin-2) constitute the matrilysin subfamily of MMPs and are expressed in normal and neoplastic epithelial tissue (144). Of these proteins, MMP-7 in particular is involved in broad proteolytic activity with the ability to degrade fibronectin, laminin, nidogen, type IV collagen and proteoglycan core proteins as well as to activate other MMPs, thereby playing various roles in tissue remodeling (137,145). In physiological conditions, MMP-7 is expressed in normal ductal and glandular epithelium, but also associates with several malignant tumors, such as gastric, esophageal, colorectal, liver and pancreatic cancer (146-151).

MMP-7 expression is usually restricted to tumor cells, whereas other MMPs are usually expressed in the stromal tissue surrounding the tumor also (146,152). During tumor progression and invasion, MMP-7 has been shown to increase the apoptosis of cells adjacent to tumor cells while at the same time inhibiting cancer cell apoptosis (147,152). Furthermore, reviews have found that MMP-7

reduces cancer cell adhesion and promotes cellular proliferation and invasion (152).

1.2.5 Membrane-type metalloproteinases

The group of MT-MMPs consist of six members: MMP-14, -15, -16, -17, -24, and -25. MMP-25, also known as membrane-type 6 metalloproteinase (MT6-MMP), was first observed in polymorphonuclear leukocytes and can degrade type I-IV collagens, gelatin, fibronectin and fibrin (153). A review by Sohail et al. reported MMP-25 expression in breast and colon cancer, and that the expression was linked to enhanced tumor invasion (154). Furthermore, studies have suggested that MMP-25 also plays a role in inflammation and cellular migration (154-156).

1.2.6 MMPs in oral squamous cell carcinoma

In OSCC, MMPs have been studied relatively widely, but the role of MMPs in this disease remains somewhat unclear. Although previous studies show negative or lower MMP expression in normal oral mucosa than in OSCC, we are aware of no studies that have examined MMP expression specifically in normal oral tongue tissue (53,157-161). Research has shown that MMPs are expressed in OSCCs at different subsites, including the tongue (51,53-55,58,157,160,162-164). de Vicente et al. studied MMP-7 expression in OSCC and observed expression only in cancer cells (58). Chuang et al., in contrast, found MMP-7 immunostaining in both tumors and the non-neoplastic buccal epithelium, but active MMP-7 was present only in tumor nests (157). Kusukawa et al. has examined MMP-3 expression and reported its expression in early-stage OSCC tissue, but not in the normal epithelium (53). They also found a correlation between high MMP-3 expression and advanced tumor size. Another study of

MMP-13 in OSCC also reported a correlation between increased expression and larger T stage (158).

A study of MMP-1, -2, -3, and -9 expression in a series of 96 patients with OSCC revealed that elevated expression of all these MMPs correlates with the mode of tumor invasion (the reference describes the grading of the mode of tumor invasion in detail) (54). In addition, MMP-1, -2, and -9 expression associated with the loss of ECMs, suggesting that tumor progression depends on the ability of tumor cells to degrade ECM (54). MMPs could thus play an important role in the invasion of OSCC. Kusukawa et al. reported a similar finding in early-stage OSCC in which high MMP-3 expression correlated with deeper invasion (53). Another study consisting of SCC of the tongue and the lower lip found that overexpression of MMP-7 associated with high-grade disease (163).

In OSCC, MMP-1, -2, -3, and -9 overexpression has shown a positive correlation with nodal status (53,54,160). Furthermore, Kurahara et al. observed TIMP-1 overexpression in metastatic cases of OSCC. Similarly, Hong et al. found a correlation between MMP-9 overexpression and metastasis in OSCC, whereas MMP-2 expression showed no such correlation (160). The authors suggested that MMP-9 may play a more important role in the metastasis of OSCC than does MMP-2. Aparna et al. also reported a correlation between increased MMP-2 and -9 expression and regional and distant metastasis in early-stage OTSCC (164). Furthermore, another study found an association between MMP-13 overexpression and the presence of lymph node metastases in OSCC (158).

The same study by Aparna et al. found that overexpression of both MMP- 2 and -9 correlated with local recurrence and shorter survival in OTSCC, which is in accordance with a study by Yoshizaki et al., who found

that MMP-2 overexpression correlated with local, nodal and distant metastatic tumor recurrence as well with shorter DFS (55). Likewise, MMP-9 expression appeared to predict tumor metastases and shorter DSS in early-stage (T1N0M0 and T2N0M0) OSCC (162). In addition, a recent study by Vincent-Chong et al. reported an association between MMP-13 overexpression and poor survival in OSCC (158).

As pointed out previously, MMPs have also been linked to improved survival among cancer patients. Korpi et al. made an interesting observation of MMP-8 expression and prognosis in OTSCC of different stages (51). Overexpression of MMP-8 associated with improved survival, and the tendency was particularly prominent among women, suggesting that the possible protective role of MMP-8 in OTSCC progression could be related to estrogen.

1.2.7 MMPs in head and neck cancer of other subsites

Researchers have shown that HNCs, as well as cancers outside the oral cavity, express different MMPs, but because studies include cancers of different subsites and stages, their results are difficult to compare (53-56,75,165-171).

A study by O-Charoenrat et al. found a correlation between MMP-9 expression and advanced T-stage in HNSCC of different subsites (oral cavity, oropharynx, hypopharynx, larynx) (165). Other studies have shown that overexpression of MMPs to plays a role in the invasion of HNSCC at different subsites (53-56,58,158). MMP-9 (165) and MMP-13 (56) expression reportedly correlate with infiltrative patterns of growth in HNSCC. In addition, other studies have found that MMP-13 plays a relevant role in the progression and invasion of other invasive malignancies (172,173). In LSCC, a study by Cazorla et al. found that a well-differentiated

histology correlated with high MMP-13 expression (174).

Studies have shown that overexpression of MMPs plays a role in the invasion and metastasis of HNSCC (53-56). In a study by O-Charoenrat et al. of carcinomas at different H&N subsites (oral cavity, oropharynx, hypopharynx, larynx), MMP-9 overexpression correlated strongly with lymph node involvement, whereas MMP-2 expression correlated only weakly with lymph node status (165). Furthermore, one study of MMP-13 in HNSCC patients showed that upregulation of MMP-13 serum levels correlated with lymph node metastasis (175). In Stage I-IV LSCC, TIMP-3 expression correlated with the presence of lymph node metastases and MMP-2 expression (171).

In HNSCC, MMP-7 overexpression has been linked to shorter survival and the presence of nodal metastases (58,59). In salivary gland carcinoma, low MMP-7 and high MMP-13 expression correlated with poor survival (176,177). Furthermore, MMP-13 overexpression associated with poor survival in Stage I-IV HNSCC (57). Both MMP-2 and -9 expression have been reported to associate with shorter DSS and DFS in HNSCC (169,170). Pradhan-Palikhe et al. reported that plasma levels of TIMP-2, but not of MMP-8, predicted poor survival in HNSCC (178).

1.3 TOLL-LIKE RECEPTORS

1.3.1 TLRs in immune defence and cancer

Toll like receptors (TLRs) are pattern-recognition receptors expressed by cells of the immune system and epithelial cells located near the host-environment boundary. TLRs are also present in many types of cancer, and multiple factors affect the variable and complex function of each TLR (60).

In humans, ten different TLRs have been identified, each recognizing a specific microbial component (179). All TLRs, except TLR-3, activate the MyD88 (myeloid differentiation primary response gene 88) signaling pathway, which subsequently activates downstream transcription factors, such as nuclear factor kappaB (NF-κB), and mitogen-associated protein (MAP) kinase signaling pathways, leading ultimately to the expression of a wide variety of proinflammatory cytokines, chemokines, growth factors, collagenases, and antiapoptotic proteins (60,61,179).

Researchers have identified two kinds of ligands for TLRs: exogenous pathogen-associated molecular patterns (PAMPs), which are components of microbes, and endogenous damage-associated molecular patterns (DAMPs) released from injured or inflamed tissues (60). Thus, the absence of foreign pathogens could elicite a TLR-mediated immune response.

TLR-2 and TLR-4 recognize lipopolysaccharide (LPS, a membrane component of Gram-negative bacteria), TLR-5 recognizes bacterial flagellin, TLR-7 recognizes double- or single-stranded RNA, and TLR-9 recognizes bacterial DNA (63,180). The location of each TLR in the cell is based on its function: TLRs detecting bacterial LPS and lipoproteins are normally located on the cell surface (TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6), whereas TLR-3, -7, -8, and -9, which mainly recognize viral RNA and bacterial DNA, are located in endosomes and lysosomes, where these materials are processed (60).

Studies have detected TLR expression in normal oral epithelium (67,69,70,72,75,181,182) as well as in premalignant oral lesions (181,183).

Consequently, TLR activation regulates innate and adaptive immune responses (60). The actual role of TLRs in tumorigenesis remains controversial, as they are related to both cancer progression and inhibition (61-63). Tumor progression seems to result from TLR activation in tumor cells, whereas tumor regression associates with the activation of host immune responses (63). NF-κB activation occurs in most tumor cells, and TLRs are presumably among the major activators of this pathway (61).

The relationship between inflammation and tumorigenesis has recently become widely accepted; estimates indicate that 20% of all cancer-related deaths are associated with chronic infection and inflammation (61). Studies indicate that tumor cells acquire many properties characteristic of immune cells, which allows them to communicate and regulate the immune system to promote their own survival and growth (61,116). Previous studies have demonstrated that TLR agonists may have potent immunostimulatory effects in vivo as a result of triggering *de novo* or boosting pre-existing (natural or therapy-elicited) anti-cancer immune responses. The FDA has licensed three TLR agonists for use in cancer patients: bacillus Calmette-Guérin (BCG), which operates as a mixed TLR-2/-4 agonist; monophosphoryl lipid A (MPL), a potent agonist of TLR-4; and imiquimod, a synthetic TLR-7 agonist (184). Imiquimod widely serves in the nonsurgical treatment for basal cell carcinoma (185,186). However, a precise elucidation of TLR-activated cell-intrinsic and cell-extrinsic signaling pathways, and also identification of biological markers that predict the propensity of an individual patient to benefit from TLR agonists, are needed (184).

Researchers have studied the expression of different TLRs in various cancers, including lung, prostate, breast, skin, and follicular thyroid carcinoma, as well as several gastrointestinal and gynecological cancers (187-193). These studies have shown that TLR expression serves both pro- and anti-tumor functions.

1.3.2 TLRs in oral squamous cell carcinoma

Numerous studies have shown that the HNSCCs of different subsites express TLR-2, -3, -4, -5, -7, and -9 both *in vitro* (66,71,74-76,182,194-196) and *in vivo* (65-77).

Studies have also shown that TLRs contribute to the progression of OSCC. Ng et al. studied TLR-2 expression in OSCC and hyperplastic/dysplastic oral lesions (183). Cells (chronic inflammatory cells, endothelial cells) of the OSCC microenvironment and dysplasia showed clearly higher TLR-2 expression than did cells of hyperplasia. TLR-2 expression occurred on the keratinocytes of dysplastic epithelium and OSCC, but not on the keratinocytes of hyperplastic samples. Positive TLR-2 expression in the microenvironment suggests that immune surveillance is activated against altered cells, whereas TLR-2 expression by malignant keratonicytes may correlate with apoptosis resistance and the survival of tumor cells.

A recent investigation found higher TLR-3 expression in both OSCC cells and tissue samples than in normal oral epithelial tissue (72). Luo et al. found that TLR-3 activation induces apoptosis of OSCC cells and that the administration of TLR-3 agonist inhibits OSCC tumor growth *in vivo* in a murine model (72). They concluded that TLR-3 has anticancer effects in OSCC. He et al. also investigated the expression and function of TLR-3 in OSCC (66). TLR-3 was expressed in both the OSCC tissue and the two OSCC cell lines examined. Activation of TLR-3 with lyophilized polyinosinic-polycytidylic acid (Poly(I:C)) upregulated cytokine expression, reduced cell viability by suppressing cell proliferation and inducing apoptosis, and limited cell migration in OSCC. Thus, TLR-3 activation might affect OSCC development. In contrast, Chuang et al. found that TLR-3 expression contributes to tumor invasion in

HNSCC (71). Cytoplasmic TLR-3 staining occurred in the vast majority (73%) of OSCC (Stage I-IV) tissues, and strong expression correlated with poor differentiation and perineural invasion.

When studying Stage I-IV OSCC tissue samples, normal oral mucosa, and OSCC cell lines, Sun et al. detected weak TLR-4 expression on the normal mucosa adjacent to the tumor (182). In OSCC, TLR-4 correlated inversily with grade: expression was weak in poorly differentiated tumors. High TLR-4 expression occurred in OSCC cell lines, which showed resistance to cisplatin-mediated apoptosis after pretreatment with TLR-4 ligand LPS. The authors concluded that the development of resistance to cisplatin in human OSCC might occur through a mechanism involving TLR-4 and its signaling pathway. Suppression of TLR-4 and its signaling pathway could thus increase sensitivity to cisplatin. Szczepanski et al. studied TLR-4 in advanced-stage (III-IV) OSCC and laryngeal squamous cell carcinomas (LSCC) (75). All tumors and HNSCC cell lines showed TLR-4 expression, which occurred in as many as 9/10 of normal oral mucosa, although cytoplasmic expression of TLR-4 in non-neoplastic tissue was weaker than that in the malignant tissue. TLR-4 expression intensity correlated inversily with tumor grade: poorly differentiated tumors express little TLR-4, as the previous study by Sun et al. showed (182). TLR-4 triggering protected tumor cells from lysis mediated by NK-92 cells, but LPS binding to TLR-4 on tumor cells enhanced cell proliferation. Based on these findings, Szczepanski et al. concluded that TLR-4 expression contributes to HNSCC progression and protects the tumor from immune system attack (75).

Researchers have also examined TLR-4 and -5 in early-stage (T1-2N0M0) OSCC and cutaneous squamous cell carcinoma (CSCC) (67). TLR-4 expression was similar in OSCC and CSCC tumors, but TLR-5 expression

was more abundant in OSCC than in CSCC. TLR-5 ligand flagellin had no effect on the HSC-3 cell line, but did induce the migration and invasion of less aggressive mucocutaneous cell lines.

A study of TLR-5 expression in OTSCC found that strong expression was an independent predictor of cancer mortality and disease recurrence (70). TLR-5 expression occurred in 84 of 101 normal epithelial and in 118 of 119 OTSCC samples, and the expression was stronger in OTSCC than in normal epithelium. In OTSCC, higher TLR-5 expression associated with age > 70 years, female gender and disease recurrence. TLR-5 expression showed no association with grade, stage or treatment.

Helicobacter pylori (HP) is a flagellated, Gram-negative bacteria believed to be the most common source of chronic bacterial incfection in humans (68). Since the recognition of bacterial flagella involves TLR-5, which is thought to promote tumor growth through inflammation-dependent mechanisms in epithelial cells, Grimm et al. studied TLR-5 expression together with HP expression in Stage I-IV OSCC and in two OSCC cell lines (68). They detected HP in 21.5% of OSCCs and found that HP expression associated with reduced DFS. Multivariate analysis showed HP expression to be an independent prognostic factor in OSCC. TLR-5 expression was absent from normal oral mucosa, but overexpressed in OSCC. However, TLR-5 expression showed no correlation with either clinicopathological characteristics or survival.

A study by Ahn et al. showed that TLR-7 agonist imiquimod inhibited OSCC cell proliferation in a dose-dependent manner (195). Imiquimod also induced necrotic cell death in OSCC cells. The authors concluded that imiquimod effectively inhibited OSCC cell growth by inducing apoptosis and necrosis. Thus, imiquimod could be considered an effective therapeutic for OSCC.

A study by Kauppila et al. found that high TLR-9 expression was an independent prognostic factor in Stage I-IV OTSCC (69). TLR-9 also mediated OTSCC invasion *in vitro*. TLR-9 expression occurred in 181 of 195 OTSCC tissue samples and was higher in OTSCC than in normal epithelium. High TLR-9 expression associated with high MMP-13 expression and poor tumor differentation (grade). TLR-9 ligand CpG-ODN increased OTSCC cell invasion and migration. TLR-9 siRNA (small interfering RNA) and inhibition by TLR-9 antibodies, in contrast, reduced OTSCC cell invasion and migration. Park et al. examined whether OSCC tumor progression involve TLR signaling (196). TLR-2, -3, -4, -5, -7, and -9 expression occurred in OSCC cells, but TLR-2 and TLR-5 activation by bacterial LPS and flagellin did not affect OSCC tumor cell progression. The more recent findings of Kauppila et al. (69) confirmed the previous findings of Min et al., who also studied TLR-9 expression in Stage I-IV OSCC tissue samples and OSCC cell lines (73); both found that TLR-9 expression was higher in OSCC tissue than in adjacent normal tissue. High TLR-9 expression levels associated with tumor size, clinical stage, and a high Ki-67 positive rate in OSCC. TLR-9 expression also occurred in the OSCC cell line, and stimulation with TLR-9 agonist CpG-ODN increased OSCC cell proliferation. Ruan et al. also published another study of TLR-9 expression in the OSCC cell line and found that TLR-9 activation induced OSCC cell migration and invasion (194). Elevated MMP-2 expression, secretion and activity also occurred during treatment with TLR-9 agonist CpG-ODN. Thus, TLR-9 signaling activation could promote human oral cancer invasion with MMP-2 induction.

1.3.3 TLRs in head and neck squamous cell carcinoma of other subsites

Rydberg et al. studied TLR-2, -3, and -5 expression in LSCC, a pharyngeal squamous cell carcinoma cell line, a healthy bronchial epithelial cell line, and in primary human nasal epithelial cells (74). They detected all these TLRs in the LSCC with the weakest TLR-3 expression. The TLR staining intensity varied among the different tumour cells within the HNSCC, which indicates that SCCs comprise a heterogenic cell population. Bronchial cells expressed mainly TLR-3, and nasal epithelial cells TLR-2, -3, and -5. TLR agonists induced a significant response in HNSCC cell lines, characterized by inflammation and cell death. Normal epithelial cells showed no such response. Thus, their results indicate that, the TLR system is an important target for future anti-tumor immunotherapy. Another study of advanced-stage (III-IV) LSCC, TLR-2, -3, and -4 expression occurred in both LSCC and inflammatory cells in tumor masses and tumor stroma. This observation may partly explain how tumors escape immune system surveillance (77).

Ilmarinen et al. evaluated the malignant transformation rate of laryngeal papillomas (LPs) and the potential of TLR-2, -4, and -9 immunoexpression as indicators of increased cancer risk in these patients (65). LSCC occurred in 2.8% of patients with recurrent respiratory papillomatosis. Nuclear TLR-4 staining was lower in LPs transforming into LSCC than in LPs with no malignant transformation. High cytoplasmic TLR-4 expression was typical of moderately or poorly differentiated tumors as well as of more the advanced T stage and, thus, may be associated with more aggressive disease. Cytoplasmic TLR-9 expression was lower in LPs than in LSCC.

Pries et al. studied all human TLRs (TLR-1-10) in HNSCC tissue samples from different anatomical sites (pharynx, tongue, larynx, oral cavity) as well as in tumor-draining lymph nodes, tonsillar tissue, healthy nasal mucosa, normal oropharyngeal mucosa and eight HNSCC cell lines (76). All HNSCC cell lines and 80% of solid tumors (primary tumors and lymph node metastases) expressed only TLR-3 as a predominantly intracellular protein while no other TLRs were expressed. TLR-3 expression occurred only in malignant cells and strongly associated with high protein levels and activity of transcriptional activator NF-κB. Inhibition of TLR-3 expression in HNSCC cell lines reduced oncoprotein c-Myc expression and reduced cell proliferation; correspondingly, TLR-3 overexpression in murine fibroblasts resulted in c-Myc upregulation and increased cell proliferation. Their data suggested that TLR-3 contributes to the malignant phenotype leading to invasive carcinoma in HNSCC.

1.4 Human papilloma virus and p16

The surrogate marker for human papilloma virus (HPV), p16, is widely utilized in evaluating oropharyngeal carcinomas, but its role in oral carcinoma remains unclear (122,197-200). In OSCC, p16 is seldom positive (201-205).

Even though p16 serves as a surrogate marker for HPV in OPC, it does not strictly correlate with HPV positivity (122). Interestingly, in a study of 167 patients with early-stage OTSCC, patients with tumors showing p16 overexpression were at higher risk of death and disease recurrence. In addition the concordance between p16 expression and HPV infection was poor (206). Thus, based on this study by Ramshankar et al., the prognostic signifigance of p16 in OSCC differs

from that reported in OPC (200). In contrast, in another study of 25 young adults (aged 18 to 39 years) with OTSCC, p16 positivity correlated with improved relapse-free survival and overall survival (207). In the same study, p16 overexpression failed to reliably correlate with HPV positivity.

2   AIMS OF THE STUDY

The main purpose of this study was to clarify the role of MMPs (2, 7, 8, 9, 13, and 25) and TLRs (2, 4, 5, 7, and 9) in clinically early-stage oral tongue squamous cell carcinoma (OTSCC). New information and understanding of the function as well as the prognostic and clinical significance of these proteins in OTSCC could lead to more precise evaluation of the aggressiveness of the disease and enable doctors to adjust their patients' treatment more accurately in the future. Identifying patients presenting with T1-T2, N0 disease with poor prognosis who would benefit from more aggressive treatment would prove especially useful.

The specific aims of the present study were:

1. To investigate the expression of MMP-2, -7, -8, -9, -13, and -25 in clinically early-stage OTSCCs and to determine whether the expression of these proteins is associated with clinicopathological parameters or patient outcomes.
2. To examine the expression of TLR-2, -4, -5, -7, and -9 in OTSCC tumor tissue samples and their association with clinicopathological variables and survival.
3. To characterize the expression of TLR-2, -4, and -9 in primary, metastasized and recurrent OTSCCs, and to evaluate the effect of TLR-2 and -4 antagonist GIT27 on the invasion of OTSCC cells in myoma organotypic invasion assay.

3 PATIENTS AND METHODS

3.1 Patients (I, II, III, IV)

Our study comprised 73 patients with consecutive clinically T1N0M0 or T2N0M0 OTSCC patients. Keski-Säntti et al. has described the clinical management of these patients in detail (35). Patients were treated with curative intent at the Helsinki University Central Hospital, Helsinki, Finland, in 1992-2002. The patients' demographic data appear in Table 3.

Clinical data and cause of death were retrieved from patient records and Statistics Finland, the national agency for population statistics. Tumor samples from 73 patients were available for immunohistochemistry (36 males and 37 females, median age 59 years, range 23-95 years), 48% (35/73) of which were clinically classified as T1, and 52% (38/73) as T2. An experienced head and neck pathologist re-evaluated all original histological tumor specimens. According to histopathological classification, 52 tumors (71%) were classified as pT1, and 21 (29%) as pT2. Tumor invasion depth was measured from the level of the proximate normal mucosal surface.

All patients in this study received treatment according to the Finnish national guidelines for the treatment of head and neck cancer. Regarding treatment of the neck, 31 patients underwent no further treatment primarily for the neck (follow-up only), 41 patients underwent elective neck dissection, and one patient received radiation therapy without surgery. Only those patients with deeply invasive early OTSCC tumors or who had neck metastases in neck dissection specimens received post-operative radiation therapy. Thus, radiation therapy had no effect on the evaluation of tumor characteristics or on the evaluation of occult metastases, except possibly in one patient. Of all patients,

34 underwent post-operative radiotherapy, including neck in 33 patients.

Altogether 24 (33%) patients had occult neck metastases (i.e., lymph node metastases) in elective neck dissection specimens (n = 15) or neck metastasis during follow-up without failure at the primary site (n = 9). During follow-up, ten patients developed local recurrences. Only two patients were diagnosed with distant metastases, both after a locoregional recurrence. Eleven patients had a second primary tumor. All but one patient had a minimum follow-up time of five years or until death (median 7.6 years, range 0.3-17.2), during which time 19 patients died of tongue cancer and 22 of other causes.

Study IV

To examine TLR expression in metastatic and recurrent disease, for Study IV we selected patients with occult neck metastases in END at the time of diagnosis, or the appearance of metastatic disease during follow-up, or a recurrent primary tumor (n = 30). In nine cases, recurrent tumors were inoperable. Tumor samples from 21 patients were available for immunohistochemistry (21 primary tumors, 10 occult neck metastases in END, 5 neck metastases during follow-up (without previous treatment of the neck or failure at the primary site), 7 local recurrent tumors and 3 recurrent tumors of the neck. This subset of patients included 11 men and 10 women (median age 56 years, range 37-83). Of these tumors, 7 (33%) were initially clinically classified as T1, and 14 (67%) as T2. Histopathological evaluation classified 11 (52%) primary tumors as pT1, and 10 (48%) as pT2. Regarding primary surgical treatment, 8 patients underwent no further treatment to the neck and 13 patients underwent elective

neck dissection; 16 patients had occult neck metastases (i.e., lymph node metastases in the END specimen (n = 11) or the appearance of neck metastasis during follow-up without failure at the primary site (n = 5)). During follow-up, 8 of these patients developed local recurrence, 14 died of tongue cancer, and 4 of other causes.

Table 3. Patient-related and clinopathological features of the 73 patients with early-stage (clinically T1, N0 and T2, N0) oral tongue squamous cell carcinoma.

Clinocopathological variable	No of patients (%)
Sex	
Male	36 (49)
Female	37 (51)
Age, years	
≤ 60	40 (55)
> 60	33 (45)
Range	23-95
Median	59
Invasion depth (mm)	
≤ 4	29 (40)
> 4	44 (60)
Grade	
I	24 (33)
II	35 (48)
III	14 (19)
Clinical T Stage	
cT1 (≤ 20 mm)	35 (48)
cT2 (21-40 mm)	38 (52)
Pathological T Stage	
pT1 (≤ 20 mm)	52 (71)
pT2 (21-40 mm)	21 (29)
Pathological node positivity*	
pN0	26 (36)
pN+	15 (21)
Pathological Stage	
I	46 (63)
II	12 (16)
III	12 (16)
IV	3 (4)

* 41/73 (56%) of the patients underwent elective neck dissection

3.2   Tissue microarray (I, II, III)

Tissue microarray (TMA) blocks were prepared from primary tumors. For TMA blocks, three different invasion depth areas (surface, center, invasive front) from H&E stained slides were selected. From marked areas, six representative 1-mm cores from each tumor were detached and placed in a paraffin block with a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA) as described elsewhere (208,209). From all samples, two similar blocks were produced. In the final analysis of each immunostaining representative, tissue was lacking for two to ten patients (for details, see the original articles).

3.3   Immunohistochemistry (I, II, III, IV)

Paraffin blocks were cut into 4- to 5-μm sections. These sections were then deparaffinized in xylene and rehydrated through a series of graded alcohol. The slides were treated in a PreTreatment module (LabVision Corp., UK Ltd., UK) with Tris-HCl buffer (pH 8.5) (all MMPs and TLRs 2, 4, 5, and 7) or EDTA buffer (pH 9.0) (TLR-9) for 20 min at 98°C, followed by endogenous peroxidase blocking for 5 min with 0.3% Dako REAL Peroxidase-Blocking Solution. The immunostaining procedure was performed by an Autostainer 480 (Lab Vision Corp.) with primary antibodies (Table 4) for one hour (I, II, III), except in Study IV, where staining took place manually, and primary antibodies were added for 16 to 21 hours. This was followed by a 30-min incubation with a Dako REAL EnVision/HRP detection system and Rabbit/ Mouse (ENV) reagent. Dako REAL DAB+ Chromogen finally served to visualize the slides for 10 min. Between each step, the slides were washed with PBS-0.04%-Tween20. Slides

were counterstained with Meyer's hematoxylin and mounted in mounting medium (Aquamount, BDH, Poole, UK). Tissues known to be highly positive for the proteins under study served as positive controls, and for negative controls, the specimens were processed with no primary antibody. The staining protocol for Ki-67 appears in Häyry et al. (126). In addition, we carried out MMP-7 immunostaining of non-neoplastic tongue tissue from six patients (II), and TLR immunostainings from ten patients (III).

3.4 Scoring of Immunohistochemical stainings (I, II, III, IV)

Two independent researchers in each study (J.H. and F.P-S. in Studies I, II; L.K.M. and J.H. in Studies III, IV), blinded to the clinical data (I, II, III), evaluated the staining positivity of different markers by evaluating the percentage of positively stained tumor cells and, in cases of disagreement, reached consensus. In tumor cells, the immunopositivity of MMP-2, -8, and -9 was cytoplasmic, but that of MMP-13 was nuclear. Grading in Study I was carried out as follows: 0 = no positivity, 1 = up to 10% positive tumor cells (low), 2 = 11-50% (moderate), 3 = 51-90% (high), 4 = over 90%, strong, intense overall positivity (very high). Concerning MMP-7 and -25, the percentage of positive tumor cells was evaluated as follows: no positivity was graded as 0, positive cells up to 20% as 1 (low), 21-50% as 2 (moderate), 51-80% as 3 (high) and over 80% as 4 (very high) (II). The cytoplasmic staining intensity of TLR-2, -5 and -9 was scored as 0 for negative, 1 for mild, 2 for moderate, and 3 for strongly

Table 4. Antibodies used and staining patterns in OTSCC.

Antigen	Primary antibody	Dilution	Staining pattern
MMP-2	NCL 507, Novocastra, Newcastle, UK, monoclonal	1:75	Cytoplasmic
MMP-7	Clone 141-7B2, Millipore, Temecula, USA, monoclonal	1:2000	Cytoplasmic
MMP-8	Hanemaaijer et al. (210) (Commercially unavailable)	1:100	Cytoplasmic
MMP-9	44236, Calbiochem, Merck KGaA, Darmstadt, Germany, polyclonal	1:1000	Cytoplasmic
MMP-13	IM44, Calbiochem, Merck KGaA, Darmstadt, Germany, monoclonal	1:50	Nuclear
MMP-25	M4942, Sigma, St Louis, USA, polyclonal	1:300	Cytoplasmic
TLR-2	H-175, Santa Cruz Biotechnology, Inc, CA, USA, polyclonal	1:50	Cytoplasmic and/or nuclear
TLR-4	H-80, Santa Cruz Biotechnology, Inc, CA, USA, polyclonal	1:50	Cytoplasmic and/or nuclear
TLR-5	IMG-664A, Imgenex, San Diego, CA, USA, monoclonal	1:200	Cytoplasmic
TLR-7	IMG-581A, Imgenex, San Diego, CA, USA, polyclonal	1:300	Nuclear membranous
TLR-9	H-100, Santa Cruz Biotechnology, Inc, CA, USA, polyclonal	1:100	Cytoplasmic
p16INK4a	Clone E6H4, 9511, CINtech® Histology Kit, Roche, Germany, monoclonal	Ready-to-use	Cytoplasmic

positive. Nuclear TLR-2 staining, cytoplasmic TLR-4 staining, and nuclear membranous TLR-7 staining was scored according to the percentage of positive tumor cells as follows: 0 = no positive cells, 1 = positive cells up to 10% (low), 2 = 11-50% (moderate), 3 = 51-80% (high), and 4 = over 80% (very high). p16^{INK4a} was classified as positive when over 70% of the cells were stained. Immunoscore of the primary tumors was evaluated from the surface and invasive front of the tumor. In organotypic myoma invasion assay, TLR-2, -4, and -9 staining positivity was scored in same manner as for OTSCC tumor tissue samples (A.A.).

On the tissue array slides, six specimens from each tumor were evaluated, and, for each case, the highest immunoscore was selected for analysis (I, II, III).

3.5   Cell culture and preparation of total cell lysates (III, IV)

Human oral tongue cancer cell line (HSC-3, JCRB0623), received from the Institute of Dentistry, University of Helsinki, was propagated in an equivalent amount of DMEM medium with 4.5 g/l glucose and Ham's F12 Nutrient mixture supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin, as well as 1 mM sodium pyruvate, 250 ng/ml fungizone and 0.4 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO). Cells were lysed in Nonidet P-40 (NP-40) lysis buffer (1% NP-40, 20 mM HEPES, pH 7.5, 150 mM NaCl) supplemented with 50 mM NaF, 1 mM Na3VO4 and 1 x Complete Proteinase Inhibitor Cocktail (Roche, Basel, Switzerland) at 4°C for 30 min. Detergent-insoluble material was removed by centrifugation (16 000 x g at 4°C for 15 min). Protein concentrations were measured

by using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

3.6   Western immunoblot (III)

Fifty μg of total cell lysates were separated with 10% SDS-PAGE, transferred to PVDF-FL membranes (Millipore, Billerica, MA) and blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) diluted to 1:1 with PBS. The membranes were incubated with rabbit anti-TLR-2 (Santa Cruz Biotechnology, Inc.), rabbit anti-TLR-4 (Santa Cruz Biotechnology, Inc.) and mouse anti-actin (Sigma) IgGs, followed by Alexa Fluor 680 (Invitrogen) and IRDye 800 (LI-COR Biosciences) anti-mouse or anti-rabbit IgGs. The signal was detected using an Odyssey Infrared Imager (LI-COR) and subsequently quantified using Odyssey software.

3.7   Myoma organotypic invasion assay and GIT27 (IV)

To study the effect of TLR-2 and -4 antagonist GIT27 (Tocris Bioscience, Bristol, UK) on the invasion of the OTSCC cell line (HSC-3), we used organotypic myoma invasion assay. Myoma disks were preprared from uterine leiomyoma. Disks were placed into transwell inserts and on top of each myoma 800 000 cells were added. After 24h, the myomas were transferred to 12-well plates on a nylon disk resting on steel grids. Normal cell culture media without GIT27, 10 μg/ml or 30 μg/ml GIT27, was used. Media were changed every 3-4 days, the myomas fixed in formalin after 14 days, and embedded in paraffin. The method is explained in more detail by Nurmenniemi et al. (211).

3.8   Statistical methods

We used SPSS 22.0 software (IBM Corporation, NY, USA) and Prism 6 (GraphPad Software Inc, San Diego, CA, USA) for the statistical analyses. A two-sided *P*-value of less than 0.05 was considered statistically significant. Immunoexpression scores of MMPs and TLRs were compared with clinicopathological variables: size (diameter and pT classification), depth of invasion, tumor grade, the presence of occult neck metastases, and patients' age and sex. Immunoexpression scores were dichotomized when appropriate. Spearman's correlation and Fisher's exact test or the χ2 test served to analyse correlations between categorical variables. Similarly, the scores of TLR stainings were compared with p16^{INK4a} staining scores (III). MMP-7 served as an explanatory variable in a logistic regression model to analyse the association between MMP-7, invasion depth and occult neck metastases. A proportional odds model served to analyse the association between MMP-7 and grade. Ki-67 expression was analyzed as described by Häyry (126).

In Study IV, TLR immunoexpression scores of primary tumors were compared to the TLR expression scores of metastases and recurrent tumors. The non-parametric Mann-Whitney U test served to analyze statistical differencies between TLR staining patterns. Parametric tests (unpaired *t*-test) served to test statistical significances of *in vitro* tests.

The Kaplan-Meier estimates, the log-rank test and a multivariate Cox regression model served to evaluate the immunoexpression scores of MMPs and TLRs. DSS time was defined as the interval between the date of the first treatment (surgery) and death from tongue cancer or the end of follow-up. DFS time was calculated from the date of the first treatment to the first recurrence of the disease.

A multivariate analysis (Cox regression) performed in Study III included the following variables: pT-stage, grade, presence of occult neck metastases, invasion, and TLR-5 immunoscores. Variables were selected in a backward stepwise manner, and a *p*-value of 0.05 served as the limit for inclusion of a covariate. Also, covariates previously shown to have prognostic value in OTSCC were selected.

3.9   Ethical conciderations

The National Supervisory Authority of Welfare and Health, and the Research Ethics Committee at the Helsinki University Central Hospital approved the study design (Dnro 166/E9/07). Because this retrospective study did not affect the treatment of the patients, nor were the individual patients identifiable from the research, the study design required no informed consent.

4   RESULTS

4.1   Matrix metalloproteinases (I, II)

4.1.1   MMP expression in OTSCC

The majority of OTSCCs expressed all of the MMPs under study (staining patterns appear in Figure 1). The score distributions of different MMPs in OTSCC appear in Table 5.

Tumor tissue material was available from 71 patients for MMP-25 staining and from 70 patients for other MMPs under study. MMP-2 staining showed that the frequency of immunopositive cells varied from low to very high. Most of the tumors (76%, 53/70) exhibited low or moderate MMP-2 immunopositivity. MMP-7 positivity was low (35/70, 50%) or moderate (21/70, 30%) in the majority of tumors, and only seven tumors had a high frequency of positive cells (7/70, 10%). None of the samples showed very high immunopositivity. MMP-8 expression was high or very high in 74% (52/70) of the tumors. None of the tumors showed very high MMP-9 frequency. Most tumors (76%, 53/70) showed low or moderate MMP-9 immunopositivity. MMP-13 expression was high or very high in the majority (77%, 54/70) of tumors and MMP-25 immunopositivity ranged from low to very high.

None of the normal tongue tissue samples showed MMP-7 immunopositivity.

Table 5. Score distribution of six different MMPs in OTSCC (n (%)).

	None = 0	Low = 1	Moderate = 2	High = 3	Very high = 4	Total positivity (%)
MMP-2	11 (16)	39 (56)	14 (20)	4 (6)	2 (3)	59/70 (84)
MMP-7	7 (10)	35 (50)	21 (30)	7 (10)	0	63/70 (90)
MMP-8	2 (3)	5 (7)	11 (16)	30 (43)	22 (31)	68/70 (97)
MMP-9	12 (17)	22 (31)	31 (44)	5 (7)	0 (0)	58/70 (83)
MMP-13	4 (6)	2 (3)	10 (14)	30 (43)	24 (34)	66/70 (94)
MMP-25	7 (10)	8 (11)	17 (24)	30 (42)	9 (13)	64/71 (90)
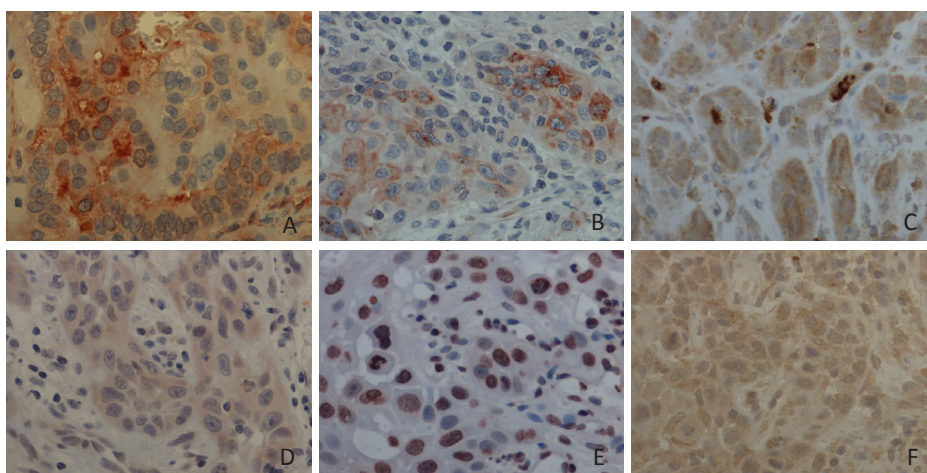
Figure 1. Immunohistochemical staining of MMP-2 (A), -7 (B) -8 (C), -9 (D), -13 (E), and -25 (F) in oral tongue squamous cell carcinoma (x 60). MMP-13 expression is nuclear, and the expression of other MMPs under study occurs in the cell cytoplasm. Image: Jaana Hagström.

4.1.2 Correlations between MMPs and clinicopathological variables

All correlations between MMPs and clinicopathological variables appear in Table 6.

MMP-7 expression predicted a higher probability of occult cervical metastases (OR 3.67, 95% CI 1.29-10.43, p = 0.013) as well as greater invasion depth. In tumors with an invasion depth over 4 mm, MMP-7 expression was usually high, whereas in tumors with a lower invasion depth, MMP-7 expression was correspondingly low (OR 4.60, 95% CI 1.47-14.39, p = 0.005).

High MMP-7 expression was associated with histologically poorly differentiated tumors (OR 3.30, 95% CI 1.27-8.57, p = 0.007, proportional odds model). We found no correlation between MMP-7 expression and tumor size (T stage), patient age or sex.

MMP-13 expression correlated with invasion depth (*P* = 0.017, Spearman's correlation, *P* = 0.042, Fisher's exact test) as well as with pT class (*P* = 0.008, Spearman's correlation). MMP-13 expression showed no correlation with tumor grade or neck metastases, Ki-67-score, or patient's sex or age.

MMP-2, -8, -9 and -25 expression failed to correlate with any tumor or patient-related parameters.

4.1.3 Prognostic aspects of MMPs in OTSCC

MMP-7 expression predicted poor overall survival (OS) (p = 0.021) (Figure 2). Even though the Kaplan-Meier plot suggested a potential difference in disease-specific survival (DSS) between the groups, we found no statistical significance. All patients with totally negative MMP-7 staining survived throughout the follow-up period. In contrast, 57% (4/7) of patients with high MMP-7 protein expression succumbed to OTSCC during follow-up. We also evaluated the effect of MMP-7 expression on disease-free survival time, but found no correlation.

Table 6. Association of MMP and TLR expressions with clinicopathological variables in OTSCC patients (p-value).

	MMP-2	MMP-7	MMP-8	MMP-9	MMP-13	MMP-25	TLR-2(c)	TLR-2(n)	TLR-4	TLR-5	TLR-7	TLR-9
Age (years, continuous variable)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sex	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Grade (I-III)	NS	0.007[c]	NS	NS	NS	NS	0.021[a]	NS	0.005[a]	0.039[a]	NS	0.003[a]
Tumor size (mm, continuous variable)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Tumor size (pT1 ≤ 20 mm, pT2 21-40 mm)	NS	NS	NS	NS	0.008[a]	NS	NS	NS	NS	NS	NS	NS
Invasion depth (mm, continuous variable)	ND	0.005[b]	ND	ND	ND	ND	0.026[a]	NS	0.008[a]	NS	NS	0.019[a]
Invasion depth (categorical, 0-4, > 4 mm)	NS	NS	NS	NS	0.041[a]	NS	NS	NS	0.018[a]	NS	NS	0.012[a]
Occult neck metastasis (N0/N+)*	NS	0.013[b]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

(c) cytoplasmic
(n) nuclear
* Lymph node positivity in pathological examination or neck metastasis during follow up without a local recurrence
[a] Spearman's rho
[b] logistic regression model
[c] proportional odds model

NS, not significant
ND, not done
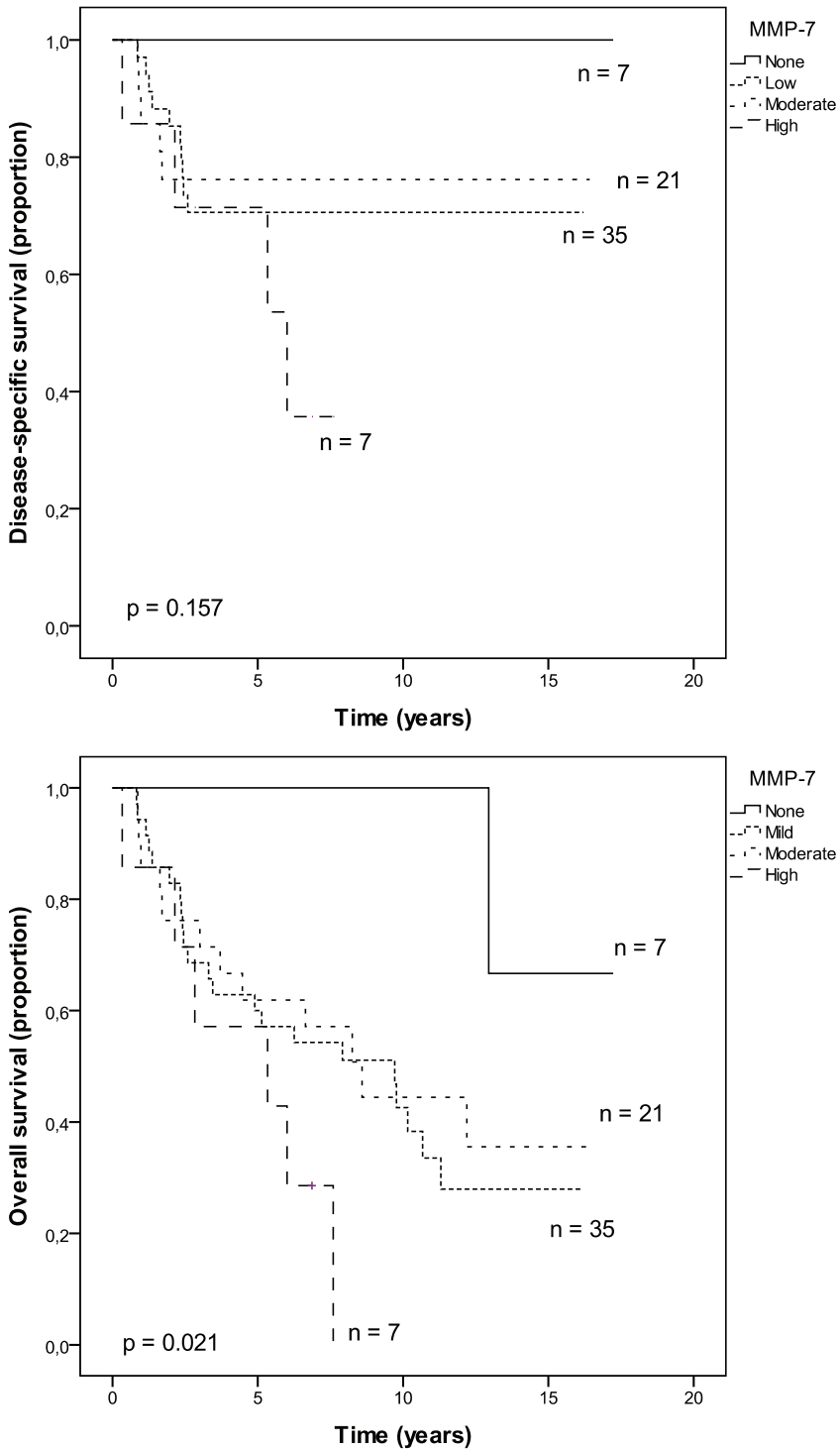
Figure 2. MMP-7 expression predicted poor overall survival (p = 0.021, log-rank test). The Kaplan-Meier plot suggested a potential difference in disease-specific survival between the groups, but we found no statistical significance.
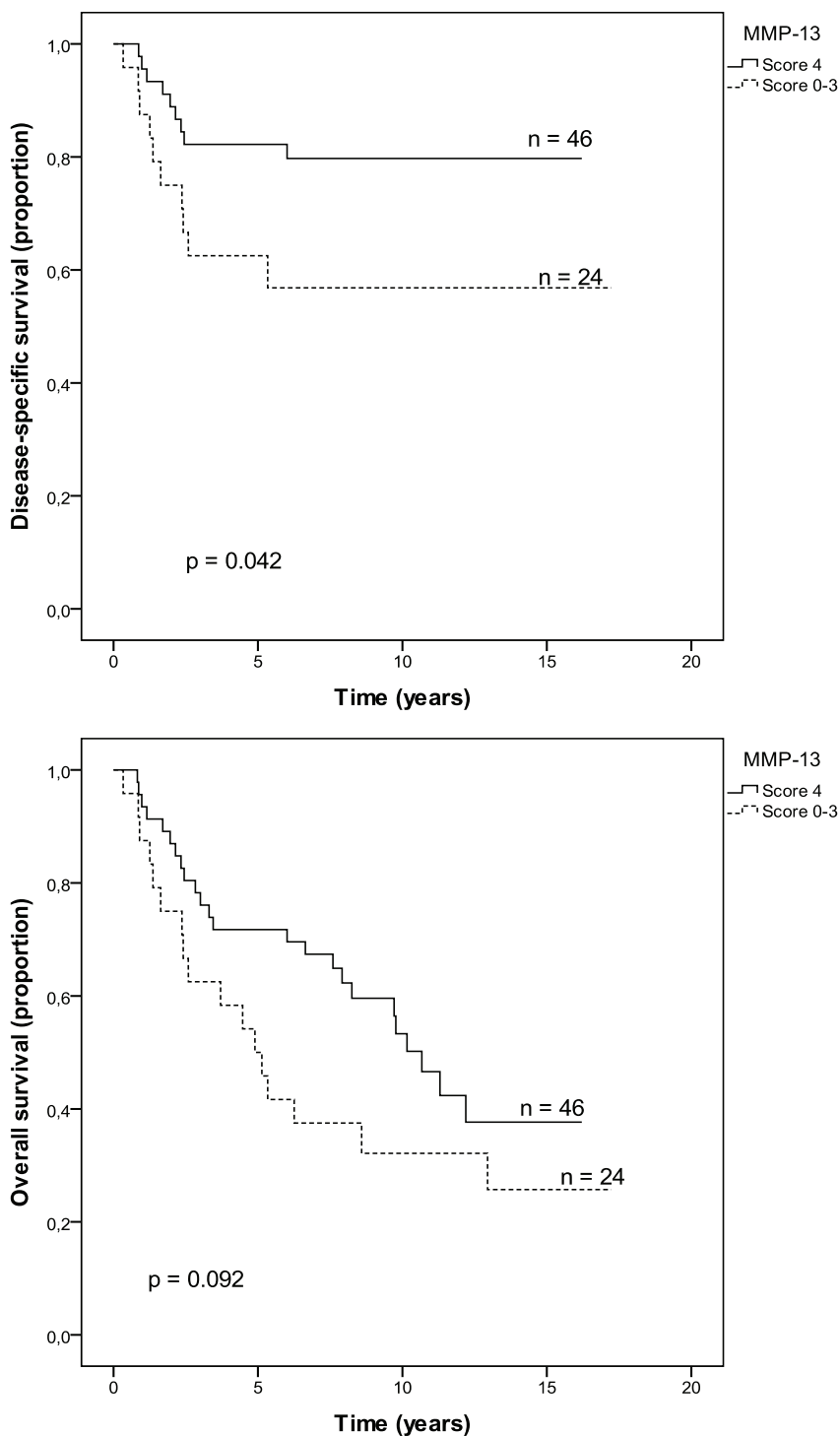
Figure 3. A Kaplan-Meier plot showing a significant difference (p = 0.042, log-rank test) in the disease-specific survival of patients with no to high (scores 0–3) (dotted line) and very high (score 4) (solid line) MMP-13 expression. The difference between these two groups in overall survival was not significant.

MMP-8 expression showed no correlation with any tumor or patient-related parameters, and no significant differences in outcome emerged. However, the DFS time was shorter in groups with negative (n = 2) and very strong (n = 22) MMP-8 expression than in those with low to high (n = 46) immunoexpression, although this difference failed to reach significance (data not shown).

Very high MMP-13 expression predicted poor DSS (very high intensity vs. others, p = 0.042, log-rank test, Figure 3). The five-year DSS of patients with no to high MMP-13 expression was 82% (95% CI 71-93%), compared to 63% (95% CI 43-83%) for patients with very high MMP-13 expression, as the Kaplan-Meier plot also showed. The correlation with DFS time failed to reach statistical significance.

The expression of gelatinases MMP-2 and -9 or membrane-type MMP MMP-25 showed no correlation with any prognostic variables.

4.2 Toll-like receptors (III, IV)

4.2.1 TLR expression in OTSCC (III, IV)

All the different TLRs examined were expressed in OTSCC tissue (staining patterns appear in Figure 4). Figure 5 shows their score distribution in tissue array samples (III) in detail. The majority of the tumors showed moderate or strong/high or very high expression of nuclear and cytoplasmic TLR-2 and cytoplasmic TLR-4. TLR-5 immunoexpression was moderate or strong in most of the tumors. The nuclear membranous positivity of TLR-7 was present mostly at a low or moderate level. TLR-9 expression occurred mainly at a mild or moderate level. Only six tumors showed strong TLR-9 expression.

Most primary and recurrent tumors and neck metastases expressed TLR-2, -4, and -9. In tissue array specimens, nuclear TLR-4 positivity occurred so seldomly that we did not
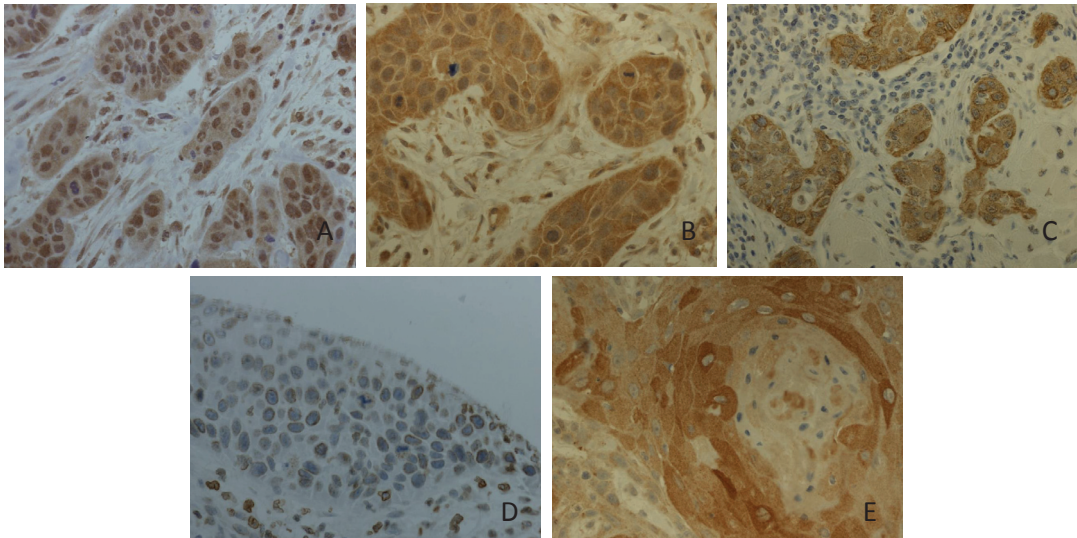
Figure 4. Immunohistochemical staining of TLR-2 (A), -4 (B), -5 (C), -7 (D), and -9 (E) in oral tongue squamous cell carcinoma (x 40). TLR-2 expression is nuclear and cytoplasmic, TLR-4 expression is mainly cytoplasmic, TLR-7 expression is nuclear membranous, and TLR-5 and -9 expression occurs in the cell cytoplasm. Image: Jaana Hagström.

score it, whereas whole tissue samples from primary tumors presented with scarce nuclear TLR-4 positivity, and was scored separately from the surface of the tumor and the invasive front. The expression of TLR-4 and -9 on the surface of the primary tumor differed from that on the invasive front. Cytoplasmic TLR-4 was stronger at the invasive front ($p < 0.001$, Mann-Whitney U-test), whereas nuclear TLR-4 expression was stronger on the surface of the tumor ($p = 0.038$, Mann-Whitney U-test). The invasive front showed stronger cytoplasmic TLR-9 expression on the surface of the tumor ($p = 0.002$, Mann-Whitney U-test).

Primary tumors expressed nuclear TLR-2 more often than did neck metastases or recurrent tumors of the neck ($p = 0.006$, Mann-Whitney U-test), whereas nuclear TLR-4 expression ($p = 0.036$, Mann-Whitney U-test) and cytoplasmic TLR-9 expression ($p = 0.08$, Mann-Whitney U-test) were higher in primary tumors than in local recurrent tumors.
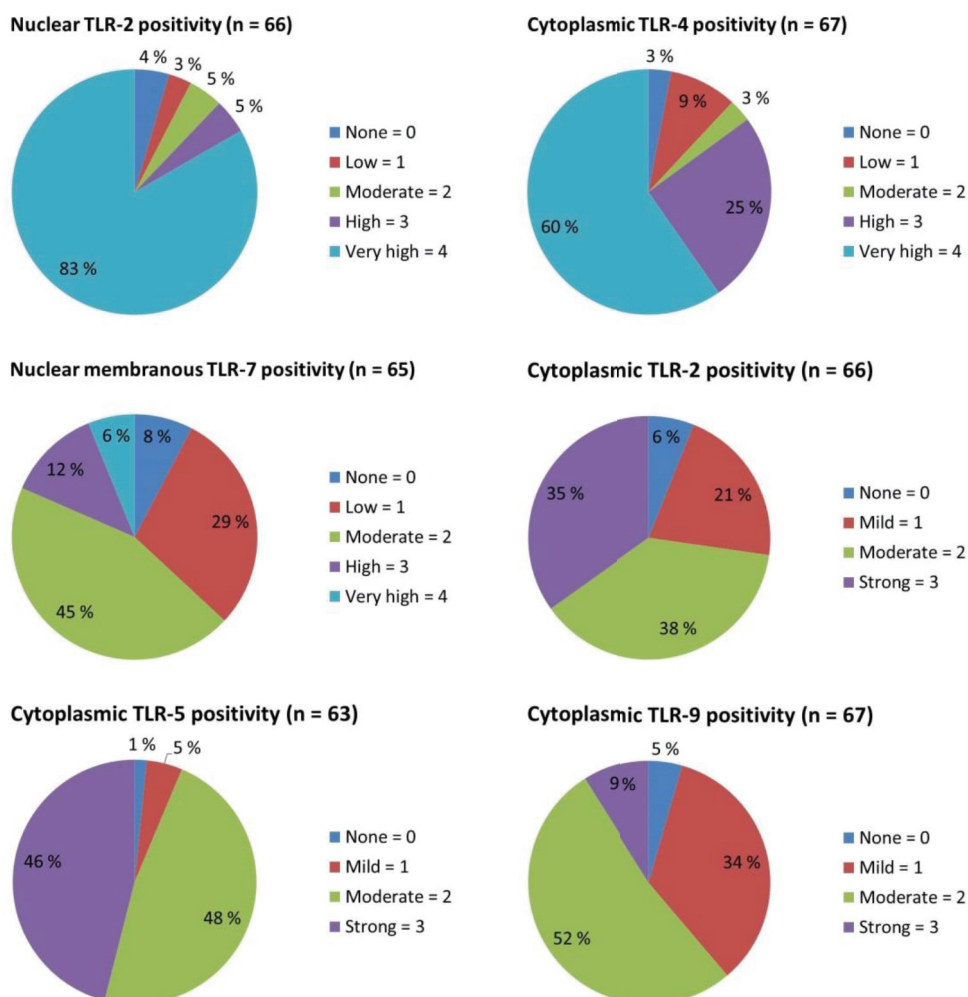
Figure 5. Score distributions of nuclear TLR-2, nuclear membranous TLR-7, and cytoplasmic TLR-2, -4, -5, and -9 expression in tissue microarray samples of oral tongue squamous cell carcinoma.

4.2.2 TLR Expression in non-neoplastic tongue tissue (III)

TLRs also occurred in non-neoplastic tongue tissue, but expression differed somewhat from that in cancer tissue. We observed TLR-2 positivity in the nucleus and cytoplasm in both non-neoplastic and carcinoma tissue, but expression was stronger in carcinomas. Nuclear expression of TLR-4 was stronger in non-neoplastic tissue samples than in OTSCC, which had such scarce TLR-4 positivity in the nucleus that we did not score it. Cytoplasmic TLR-4 positivity occurred more often in carcinoma than in benign tissue. Cytoplasmic TLR-5 immunoexpression was moderate or strong in most of the benign and malignant samples. TLR-7 was nuclear membranous in both non-neoplastic and malignant tissue. TLR-9 expression was cytoplasmic in both benign and carcinoma tissue.

4.2.3 TLR-2 and -4 in Western Blot analysis (III)

Western blot analysis confirmed that TLR-2 and -4 expression occurred in the HSC-3 tongue carcinoma cell line, and that their molecular weights corresponded with those of full-length proteins.

4.2.4 Correlations between TLRs and clinicopathological variables (III)

All correlations between TLRs and clinicopathological variables appear in Table 6.

Both high cytoplasmic TLR-2 and TLR-4 expression correlated with deeper tumor invasion ($p = 0.026$ and $p = 0.008$, respectively, Spearman's rho) and higher tumor grade ($p = 0.021$ and $p = 0.005$, Spearman's rho), whereas high TLR-5 expression correlated with lower tumor grade ($p = 0.039$, Spearman's rho). High TLR-9 expression correlated with

deeper tumor invasion ($p = 0.019$, Spearman's rho) and larger tumor diameter ($p = 0.003$, Spearman's rho). Nuclear expression of TLR-2 or membranous expression of TLR-7 failed to correlate with any of the variables.

4.2.5 Prognostic aspects of TLRs in OTSCC (III)

None of the TLRs correlated with disease recurrence. In survival analyses, negative or mild (group 0, n = 4) TLR-5 expression predicted poor DSS ($p = 0.014$, log-rank test) and poor OS ($p = 0.005$, log-rank test) (Figure 6), yet multivariate analysis failed to show TLR-5 expression as an independent prognostic factor in OTSCC. Survival analyses revealed no other significant correlations.

4.2.6 Myoma organotypic invasion assay and GIT27 (IV)

In organotypic myoma invasion model assay, GIT27 failed to inhibit the invasion of HSC-3 cells into the myoma tissue. The mean invasion depths were 2.2 mm without GIT27, 3.0 mm with 10 μg/ml GIT27, and 2.5 mm with 30 μg/ml GIT27. However, the expression levels of TLR-2 and -4 varied between the surface and invasive front. TLR-2 and -4 expression was stronger in cells invading deeper into the myoma than in cells close to the surface, which showed only minimal levels of these proteins. TLR-9 expression was consistent throughout the OTSCC cells in the myoma tissue.

4.3 p16 expression (III)

p16INK4a was positive in 9% (n = 6/65) of the OTSCC samples, and its expression failed to correlate with any of the clinicopathological factors or with TLR expressions.
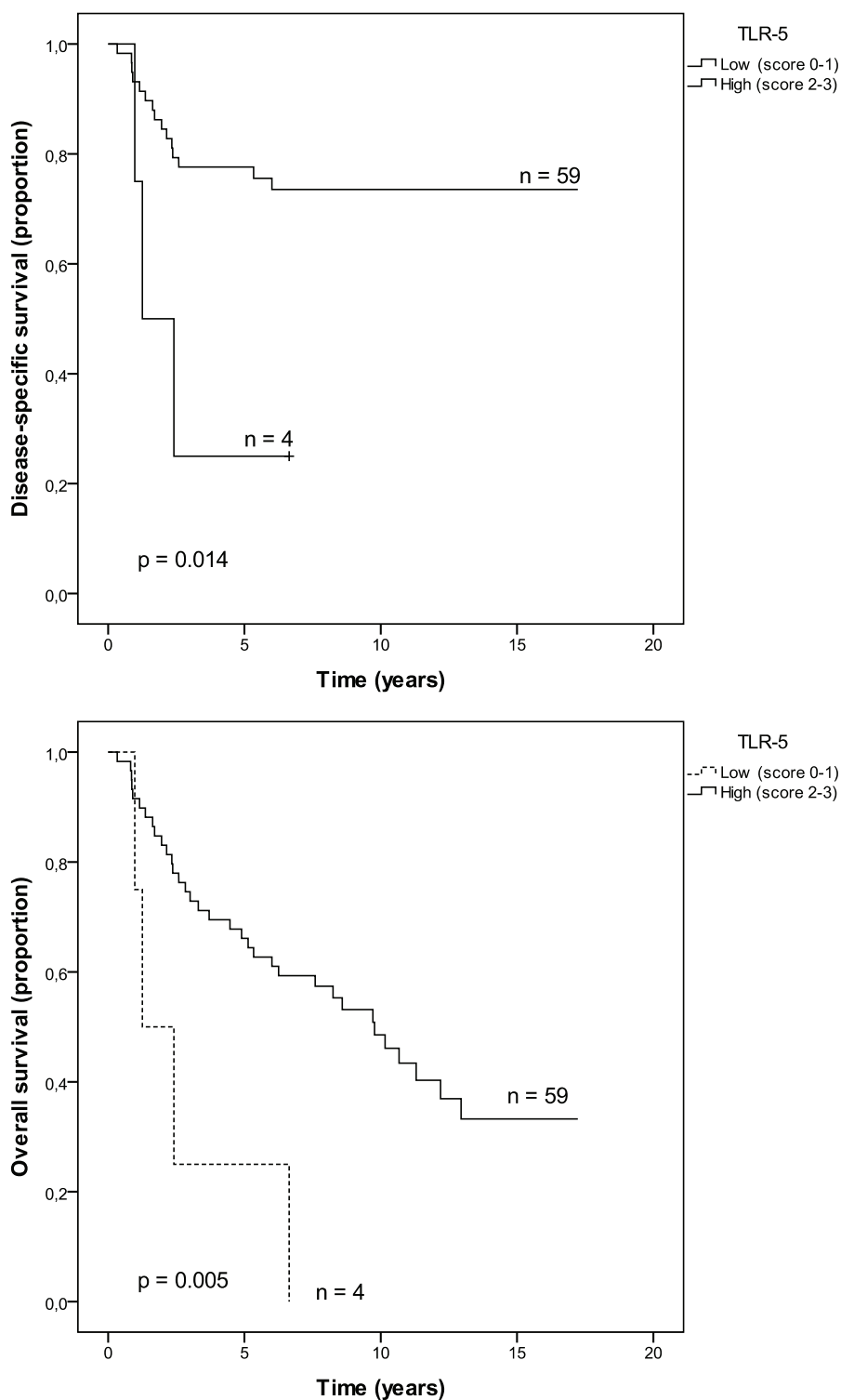
Figure 6. A Kaplan-Meier plot showing a significant difference in the disease-specific survival (p = 0.014, log-rank test) and overall survival (p = 0.005, log-rank test) of patients with negative or mild (scores 0–1) (dotted line) and moderate or high (score 2-3) (solid line) TLR-5 expression.

5   DISCUSSION

5.1   Strengths and limitations of this thesis

In this thesis, we examined the expression and clinical significance of MMPs and TLRs in a uniform sample of 73 patients with clinically early-stage OTSCC. These proteins were previously evaluated in the HNSCCs of different subsites, but many of those studies included patients in different stages of the disease. However, early- or advanced-stage OTSCC involves malignancies with different manifestations and outcomes. We included only T1-2 tongue tumors, as they can in practice always undergo surgery with adequate margins, yet many of them develop occult metastases, recur or behave aggressively. Thus, such a patient population offers a sound basis for examining differences in protein expressions and for comparing expressions with tumor behavior. It is worth noting that the pathological classification of some of our patients also indicated advanced-stage tumors, as some patients had occult metastasis in END or, if no END was performed, during the follow-up period.

Patients underwent diagnosis and treatment between 1992 and 2002, which provided a long follow-up period of at least five years. Treatment of OTSCC has changed little in recent decades, and patients in this sample received treatment according to the Finnish national treatment guidelines for head and neck cancer. For example, all patients with occult neck metastases also received post-operative radiotherapy. However, various treatment modalities may obviously constitute a confounding factor for retrospective outcome analyses. Nevertheless, even in a prospective study and population setting, a number of confounding factors, such as the

smoking habits of the patients, vary, as does the treatment of the neck. Moreover, had we selected an even more uniform patient group, the study would have had too few patients.

Studies I-III were performed using the TMA technique, which is a method for easily studying a large number of molecular markers. Furthermore, although the TMA technique requires little valuable tissue material, it has its limitations (212). Because a single TMA sample requires only a small (1 mm) punch from each area of the tumor, possible variations in protein expression in different parts of the tumor can skew evaluations of expression. This study used three core tissue biopsies from each tumor: from the tumor surface, the middle part, and the invasive front, yielding two similar TMA blocks; thus, we evaluated six histospots of each tumor. However, Studies I-III, which used the TMA technique, failed to evaluate the immunoexpression scores according to the localization of each biopsy. Evaluating differences in expression between the surface and the invasive front of the tumor was therefore impossible. Comparing expressions across different sites of the tumor was possible in Study IV, which used whole tissue slides.

The absence of data on patients' smoking and alcohol consumption could be considered a shortcoming of this study, because smoking and alcohol are the well-known risk factors in OTSCC and variables possibly affecting the survival and comorbidity of these patients. A prospective study, however, could take this absence of data into consideration.

Study IV assessed the effect of TLR-2 and -4 antagonist GIT27 on the invasion of the OTSCC cell line. Our study used only one highly invasive OTSCC cell line, so our results may not be representative of most OTSCC

cells. To draw more definite conclusions, the study should also examine some other cell lines.

5.2   MMPs in OTSCC (I, II)

MMP-7 upregulation correlated with tumor invasion, neck metastasis and grade, and predicted poor survival

In the present study, MMP-7 expression associated with invasion, metastasis, and poor OS. The findings of some previous studies of colorectal, pancreatic, and bladder cancer agree with this finding of a correlation between MMP-7 upregulation and metastatic disease (146,148,149,213). Furthermore, another study which included oral cancers of different sites and stages found that MMP-7 expression correlated with lymph node metastasis (58). Other MMPs (MMP-1, -2, -3, -7, -9, -13, -14) have also been linked to the presence of metastases in various cancers (48).

In buccal squamous cell carcinoma, overexpression of MMP-7 correlated with larger tumor size and more aggressive invasion of adjacent tissues (157). Likewise, our results showed that high MMP-7 expression associated with advanced invasion depth, whereas in our material, MMP-7 expression showed no correlation with T classification. This discrepancy may be due to previously reported differences in the behavior of OTSCC from that of OSCCs from other subsites (26,27). Furthermore, de Vicente et al. found a correlation between MMP-7 expression and invasion in OSCC from different subsites (58). However, in contrast to our results, they found no association with histological tumor grade.

In this study, MMP-7 expression predicted poor OS. However, we found no statistical significance between MMP-7 expression and DSS, although such a tendency was evident. This could be due to the small number of patients in groups with no (0%, n =

7) or high (51-80%, n = 7) MMP-7 expression. In addition, patients with tumors lacking MMP-7 expression survived throughout the follow-up period, whereas patients with high MMP-7 expression had poor DSS. Our findings call for further study to confirm the possible prognostic value of MMP-7 expression for survival.

As our present results suggest, MMP-7 expression may also have prognostic significance in other cancers, including bladder, liver, gastric, pancreatic and colorectal cancers (146,147,149,150,213-215). Another study of 29 patients with OTSCC found an association with MMP-7 overexpression and shortened survival, but the correlation was significant only among patients with positive lymph nodes (58). Weber et al. studied MMP-7 expression in tumors of the oropharynx, hypopharynx and larynx, and found that MMP-7 associates with survival in univariate, but not in multivariate analyses (59). Lequerica-Fernandez also studied MMP-7 in carcinoma of the parotid gland, but found no prognostic significance (216). In contrast to our results, Luukkaa et al. found an association between low MMP-7 expression and poor survival in acinic and mucoepidermoid carcinomas of the parotid gland (177).

This is the first study of a uniform patient material with early-stage OTSCC to demonstrate that MMP-7 overexpression associates with more aggressive disease.

MMP-13 overexpression correlated with deeper invasion and advanced tumor size and associated with poor survival

We found that MMP-13 expression to predict more aggressive tumor invasion and growth as well as poor DSS in OTSCC. Previous studies have shown that MMP-13 plays a role in tumor progression and HNSCC invasion (56),

as well as in other cancers (172,173). Marcos et al. demonstrated that upregulation of serum MMP-13 levels correlated with active MMP-13 and lymph node metastasis in HNSCC (175), which was in accordance with our results indicating that high MMP-13 expression associates with more aggressive disease.

The finding of an association between very high MMP-13 immunoexpression and poor DSS is in accordance with that of a study by Luukkaa et al., who examined stage I-IV HNSCCs from different subsites (57). They also suggested that high MMP-13 staining intensity predicts poor overall survival in salivary gland cancer (176).

With regard to disease recurrence, Luukkaa et al. previously reported a slight, but non-significant, association between MMP-13 expression and DFS in HNSCC (57). In our study, however, we found no significant correlation between MMPs and DFS either.

This study demonstrates a correlation of nuclear MMP-13 expression with invasion depth, tumor size, and DSS in early-stage OTSCC. Our results, regarding the tissue expression of MMP-13 in OTSCC strengthen and extend the previous findings of Marcos et al., who proposed that elevated serum MMP-13 levels are a potential tumor biomarker in HNSCC (175). Overall, MMP-13 may be a prognostic biomarker of tongue cancer, but verifying its role in clinical use will require further studies of tumours from other head and neck sites.

MMP-2, -8, -9, and -25 in OTSCC

In the present study, the expression of gelatinases MMP-2 and -9 correlated with none of the clinicopathological or prognostic variables.

A study by Kurahara et al. found that MMP-2 and -9 expression to correlates with regional metastasis OSCC (54). In contrast, a meta-analysis of MMPs in HNSCC reported no correlation between MMP-2 and -9 expression and nodal metastasis, which is in accordance with our results (217). O-Charoenrat et al. found a correlation between MMP-9 overexpression and advanced T-stage, an infiltrative pattern of tumor growth, and lymph node involvement, whereas MMP-2 expression and lymph node status showed only a weak correlation (165). Likewise, Hong et al. found that MMP-9 expression related significantly to metastasis in OSCC, whereas MMP-2 expression did not (160). They suggested that MMP-9 may play a more important role than MMP-2 in the metastasis of OSCC to adjacent tissues. Our study, however, failed to verify this possible influence of MMP-2 and MMP-9 on metastasis in OTSCC.

Studies by Ruokolainen et al. found that both MMP-2 and -9 associated with shorter DSS and DFS in HNSCC (169,170). Similarly, MMP-2 expression proved to correlate with tumor recurrence as well with poor DFS (55). Katayama et al. observed that MMP-9 had predictive value in metastasis and DSS in early-stage (T1N0M0 and T2N0M0) OSCC (162). However, similar to our findings, Korpi et al. found no correlation between the immunohistological staining of MMP-2 or -9 and the DSS of OTSCC patients (51).

Most of our patients presented with tumors showing high or very high MMP-8 expression. MMP-8 has reportedly acted as a protector against the spread of skin (52), breast (140), and tongue (51) cancers. However, we found no defensive effect of MMP-8 in OTSCC. It is worth noting that only two patients in our sample showed negative MMP-8 expression. Thus, we could draw no conclusion about the protective role of MMP-8. In survival analyses, patients with negative ($n = 2$) and very strong ($n = 22$) MMP-8 expression had a shorter DFS time than did patients with low to high ($n = 46$) immunoexpression, but this difference did not reach statistical significance.

We hypothesized that MMP-8 expression and its action on bioactive chemokines, cytokines, and growth factors at 'normal' levels may play a protective role in cancer (50,51,218). However, when MMP-8 is totally missing or clearly overexpressed, this protection may be lost. Very highly expressed proteins may have mutated and therefore altered their normal function.

To our knowledge, no previous studies have examined MMP-25 in HNSCC. Although some studies have reported MMP-25 expression in some malignancies (154,156), the role of MMP-25 in cancer remains inconclusive. In our patients, MMP-25 was expressed in most OTSCC tumors, but we found no correlation with clinicopahtological parameters. Its role in OTSCC therefore requires further investigation.

Conclusion

Our results on MMPs in a uniform sample of early-stage OTSCC suggest that MMP-7 overexpression could contribute to OTSCC tumor invasion and metastasis, and that upregulation of MMP-13 may predict even more aggressive tumor invasion and growth. Both MMP-7 and -13 expressions associated with poor patient survival.

5.3   TLRs in OTSCC (III, IV)

TLR expression in non-neoplastic tongue tissue

TLRs were widely expressed in OTSCC tissue, as previous studies on TLRs in HNSCC have also reported (70-77). We also evaluated TLR expression in non-neoplastic tongue tissue, in which TLRs were expressed but staining patterns differed somewhat from those in carcinoma tissue. Cytoplasmic TLR-2 expression was lower in benign tissue than in tumors, but both benign and malignant samples showed substantial nuclear TLR-2 positivity.

Ng et al. demonstrated clearly higher nuclear and cytoplasmic TLR-2 expression in the inflammatory and endothelial cells in the OSCC microenvironment, and in dysplasia, than in the hyperplastic epithelium, which suggests that altered cells trigger immune surveillance (183). In our series, TLR-4 expression was widely present in both cancer and non-neoplastic tissues, but expression was mainly nuclear in benign and cytoplasmic cancer tissue. In a previous study by Szczepanski et al., cytoplasmic TLR-4 expression was weaker in the normal epithelium than in LSCC and OSCC tissues (75). Furthermore, Sun et al. also found TLR-4 expression, although low, in normal mucosa next to OSCC (182). Strong cytoplasmic TLR-5 expression occurred in both non-neoplastic and malignant tissues in the present series, whereas Kauppila et al. found stronger cytoplasmic TLR-5 expression in OTSCC than in the normal epithelium (70). Both benign and malignant tissues in our study showed nuclear or nuclear membranous TLR-7 staining or both. To our knowledge, no previous studies have examined TLR-7 expression in OSCC tissue samples or normal oral mucosa. In our study, cytoplasmic TLR-9 was mildly or moderately expressed in both benign and malignant tissues, whereas Min et al. reported higher TLR-9 expression in OSCC than in adjacent normal mucosa (73).

TLR expression and clinocopathological variables

In our samples, TLR-2, -4, and -9 overexpression correlated with deeper tumor invasion. A study of TLR-2 in gastric cancer showed that high TLR-2 expression promoted the transcription of genes related to angiogenesis and invasion, and that expression correlated with metastasis (64). The same

study found that TLR-2 stimulation enhanced the invasive capacity of gastric cancer cells. In contrast, another study by Park et al. found no evidence of TLR-2 or -5 activation or its influence on the invasion of OSCC cells (196). Szczepanski et al. showed that TLR-4 ligand LPS enhanced HNSCC cell proliferation and that TLR-4 activation protected tumor cells from NK-92 cell-mediated lysis (75). They concluded that TLR-4 ligation facilitates HNSCC progression. Thus, our result showing a correlation between TLR-4 expression and tumor invasion is in accordance with the results of this previous study. A recent *in vitro* study by Ruan et al. demonstrated that TLR-9 activation induced the migration and invasion of OSCC cells (194), which supports our results.

In our material, TLR-2 and -4 overexpression correlated with poor tumor differentiation. In contrast, strong TLR-5 expression occurred in well-differentiated tumors. Studies have shown that TLR expression correlates with tumor grade in HNSCC: one study showed that high TLR-4 expression correlated with a low grade in LSCC (75) and OSCC (182), whereas another study showed that high TLR-3 expression correlated with poorly differentiated OSCCs (71). However, grade is not considered a very reliable prognostic predictor in OSCC, mainly because of its subjective assessment (34).

As a previous study of TLR-9 in OSCC also reported (73), in our material, high TLR-9 expression correlated with advanced tumor size. In OSCC, TLR-9 stimulation with CpG-ODN also increased tumor proliferation *in vitro* (73).

TLR expression and survival in OTSCC

Survival analyses revealed that patients with tumors showing no or mild TLR-5 expression had worse DSS than did patients with moderate or high TLR-5 expression. This

finding differs from that of Kauppila et al., who found strong TLR-5 expression to be an independent predictor of OTSCC mortality (70). However, one explanation could be that their study comprised OTSCC patients in different stages of the disease, whereas our study focused only on early-stage disease.

TLR-2 and -4 in Western immunoblot

We used Western immunoblot to study TLR-2 and -4 expression in the OTSCC cell line. TLR-2 and -4 were expressed in OTSCC cells at their expected molecular weights of 90 kDa and 91 kDa, respectively. This in an interesting finding, because studies generally report that TLR expression occurs on the cell surface (60). Thus, our results show that expression of these TLRs occurred in their full-length forms in this tongue cancer cell-line even though in cancer tissue they were located in the cytoplasm.

p16 expression in OTSCC

In these patients, we observed p16 positivity in only 9% of the tumors, which is similar to findings from previous studies of p16 in OSCC (202-204). We found no correlation between p16 expression and clinicopathological parameters or with TLR expression.

TLR expression in metastatic and recurrent OTSCC (Study IV)

For Study IV, we used whole-tissue samples to further study the role of TLRs in tumor invasion, metastases, and recurrences. In addition, we wanted to evaluate whether TLR expression on the surface of the tumor would differ from that on the invasive front.

Most primary tumors expressed TLR-2, -4, and -9 excessively. TLR expression in primary tumors differed somewhat from that in recurrent tumors or metastases. Nuclear TLR-2 expression occurred more often in primary tumors than in neck metastases or

recurrent tumors of the neck. Primary tumors showed higher nuclear TLR-4 expression and cytoplasmic TLR-9 expression than did local recurrent tumors. These findings suggest that TLRs contribute to OTSCC development and progression, but that the role of TLRs may differ in the different stages of the disease.

Sharma et al. have recently reviewed the molecular changes at the invasive front of oral cancer (219). The authors pointed out that many of the molecular events that are essential for tumor spread take place at the tumor-host interface or invasive front, where the deepest and presumably most aggressive cells exist. These events include, for example, changes in adhesion molecules, the production of proteolytic enzymes, increased cell proliferation and the triggering of angiogenesis. We also evaluated TLR expression separately at the surface of the primary tumor and at the invasive front. Cytoplasmic TLR-4 and TLR-9 expression was stronger at the invasive front than in the superficial tumor part of the tumor. In turn, stronger nuclear TLR-4 expression occurred on the tumor surface. Changes in TLR-4 expression from nuclear at the surface to cytoplasmic in the deeper parts of the tumor could possibly reflect the functional change of TLRs in tumor progression. As reported previously, TLR-2 and -4 normally occur on the cell surface, whereas TLR-9 expression usually occurs intracellularly (60). However, TLR expression may vary across different tissues from nuclear to cytoplasmic or membranous or a combination of these. In a recent review, Jouhi et al. speculated the role of different TLR expression patterns in cancer progression (220). They hypothesized that the relocation of DAMPs in the cytoplasm of cancer cells could also activate TLRs in the cytoplasm, ultimately leading to tumor progression. However, the mechanisms behind different localizations of TLRs in cells remains elusive.

Myoma organotypic invasion assay

We used myoma organotypic invasion assay to examine the invasion of the OTSCC HSC-3 cell line. TLR-2 and -4 expression was stronger in cells invading deeper into the myoma tissue, whereas TLR-9 expression was consistent throughout the tumor cells. Thus, TLRs may influence the invasion of OTSCC.

Generally, TLRs are associated with the aggressive and invasive potential of cancer. A study by Yang et al. found TLR-2 stimulation to enhance the invasive capacity of gastric cancer cells *in vitro* (64). In HNSCC, TLR-4 ligation on tumor cells has been linked to HNSCC progression (75). Furthermore, another study found TLR-9 activation to induce the migration and invasion of OSCC cells (194).

TLR-2 and -4 antagonist GIT27 did not affect the invasion of HSC-3 cells in organotypic myoma invasion assay. TLRs could utilize a different action mechanism, whether activated by DAMPs in cancer or by PAMPs in infection. Maksimovic-Ivanic et al., who studied the anti-cancer properties of GIT27 in rodent and human tumor cell lines (221), found that NO-donating isoxazole derivative GIT-27NO had anti-tumor properties and affected the viability of tumor cells, whereas the NO-deprived parental compound GIT27 had no impact on tumor cells. However, we could find no similar anti-tumor effect. One explanation for the different finding may be that the HSC-3 cell line is highly aggressive with invasive potential. Furthermore, we studied the effect of GIT27 on the invasion of tumor cells, not the viability HSC-3 cells. A previous study reported that although TLR-5 ligand flagellin did not affect the invasion of HSC-3 cells, it did induce the proliferation, migration and invasion of less-aggressive mucocutaneous cell lines (67). To our knowledge, no previous studies have examined GIT27 in HNSCC, although reports

have shown that TLR-4 ligand LPS induced proliferation and cytokine production in the OSCC cell line PCI-30, but not in another OSCC cell line, YD-10B (75,196).

Conclusion

To summarize, TLR-2, -4, -5, -7, and -9 expression occurred in OTSCC. TLR-2, -4, and -9 associated with invasive tumor growth, and expression of these TLRs occurred in primary tumors, neck metastases, and recurrent tumors. Consequently, these proteins may contribute to both the development and progression of tongue carcinoma. Changes in TLR expression from the surface of the tumor to the invasive front could reflect a functional change of TLR action in cancer progression.

5.4 Future perspectives

Our study comprised 73 OTSCC patients. Future studies should test our findings in another, preferably larger, series of tumors to further clarify the role of these proteins in OTSCC. Of particular interest should be those proteins that showed an association with tumor behavior and outcome, namely MMP-7 and -13, as well as TLR-2, -4, and -9. Identifying biomarkers that could aid in evaluating the aggressiveness and prognosis of the disease would be most useful. Such markers might permit more appropriate and individualized treatment for each patient. Clarification of the true meaning of these markers for disease survival would require a prospective randomized controlled trial.

6   CONCLUSIONS

1. MMP and TLR expression occurred in OTSCC.
2. MMP-7 overexpression associated with deeper tumor invasion, the presence of occult neck metastases, and poor OS.
3. High MMP-13 expression correlated with advanced tumor size and deeper tumor invasion, and associated with poor DSS.
4. TLR-2, -4, and -9 expression correlated with deeper tumor invasion.
5. TLR-2 and -4 antagonist GIT27 failed to inhibit the invasion of the HSC-3 OTSCC cell line in the myoma-organotypic invasion model.
6. The expression patterns of TLR-2 and -4 at the surface of the primary tumor differed from those at the invasive front, which may reflect functional changes in these TLRs during cancer progression.
7. TLR-2, -4, and -9 expression occurred in primary tumors, neck metastases, and recurrent tumors. Thus, TLRs may contribute to OTSCC in different stages of the disease.

Both MMPs and TLRs appear to affect immune defence. Environmental carcinogens (e.g., tobacco, alcohol) are well known to contribute significantly to the development of HNSCCs, whereas tumor progression could result partly from a failure of the innate immune response against cancer. Thus, the relevance of this innate immune response in cancer initiation and progression is the subject of intense research. Along with surgery, radiation, and chemotherapy, immunotherapy is likely to become the fourth means of treating cancer. How ctDNA released from tumor cells and NGS used to detect genomic alterations in tumors can serve in cancer diagnostics and treatment remains to be seen.

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REFERENCES

(1) Globocan. 2012; Available at: http://globocan.iarc.fr.

(2) Finnish Cancer Registry. 2012; Available at: www.cancerregistry.fi.

(3) Kari S, Alho OP, Jokinen K, Hyrynkangas K, Laara E. Carcinoma of the oral tongue in northern Finland: trends in overall incidence and patient and tumour characteristics. J Oral Pathol Med 26:480-483, 1997.

(4) Moore SR, Johnson NW, Pierce AM, Wilson DF. The epidemiology of tongue cancer: a review of global incidence. Oral Dis 6:75-84, 2000.

(5) Mehanna H, Paleri V, West CM, Nutting C. Head and neck cancer--Part 1: Epidemiology, presentation, and prevention. BMJ 341:c4684, 2010.

(6) Hakulinen T, Tryggvadottir L, Gislum M, Storm HH, Bray F, Klint A, Engholm G. Trends in the survival of patients diagnosed with cancers of the lip, oral cavity, and pharynx in the Nordic countries 1964-2003 followed up to the end of 2006. Acta Oncol 49:561-577, 2010.

(7) Kademani D. Oral cancer. Mayo Clin Proc 82:878-887, 2007.

(8) Argiris A, Karamouzis MV, Raben D, Ferris RL. Head and neck cancer. Lancet 371:1695-1709, 2008.

(9) Marttila E, Uittamo J, Rusanen P, Lindqvist C, Salaspuro M, Rautemaa R. Acetaldehyde production and microbial colonization in oral squamous cell carcinoma and oral lichenoid disease. Oral Surg Oral Med Oral Pathol Oral Radiol 116:61-68, 2013.

(10) Hashibe M, Brennan P, Benhamou S, Castellsague X, Chen C, Curado MP, Dal Maso L, Daudt AW, Fabianova E, Fernandez L, Wunsch-Filho V, Franceschi S, Hayes RB, Herrero R, Koifman S, La Vecchia C, Lazarus P, Levi F, Mates D, Matos E, Menezes A, Muscat J, Eluf-Neto J, Olshan AF, Rudnai P, Schwartz SM, Smith E, Sturgis EM, Szeszenia-Dabrowska N, Talamini R, Wei Q, Winn DM, Zaridze D, Zatonski W, Zhang ZF, Berthiller J, Boffetta P. Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. J Natl Cancer Inst 99:777-789, 2007.

(11) Syrjanen S, Lodi G, von Bultzingslowen I, Aliko A, Arduino P, Campisi G, Challacombe S, Ficarra G, Flaitz C, Zhou HM, Maeda H, Miller C, Jontell M. Human papillomaviruses in oral carcinoma and oral potentially malignant disorders: a systematic review. Oral Dis 17 Suppl 1:58-72, 2011.

(12) de Bree R, Haigentz M,Jr, Silver CE, Paccagnella D, Hamoir M, Hartl DM, Machiels JP, Paleri V, Rinaldo A, Shaha AR, Takes RP, Leemans CR, Ferlito A. Distant metastases from head and neck squamous cell carcinoma. Part II. Diagnosis. Oral Oncol 48:780-786, 2012.

(13) Arya S, Rane P, Deshmukh A. Oral cavity squamous cell carcinoma: role of pretreatment imaging and its influence on management. Clin Radiol 69:916-930, 2014.

(14) Peters TT, Senft A, Hoekstra OS, Castelijns JA, Witte BI, Leemans CR, de Bree R. Pretreatment screening on distant metastases and head and neck cancer patients: Validation of risk factors and influence on survival. Oral Oncol 2014.

(15) de Bree R, Takes RP, Castelijns JA, Medina JE, Stoeckli SJ, Mancuso AA, Hunt JL, Rodrigo JP, Triantafyllou A, Teymoortash A, Civantos FJ, Rinaldo A, Pitman KT, Hamoir M, Robbins KT, Silver CE, Hoekstra OS, Ferlito A. Advances in diagnostic modalities to detect occult lymph node metastases in head and neck squamous cell carcinoma. Head Neck 2014.

(16) Bessell A, Glenny AM, Furness S, Clarkson JE, Oliver R, Conway DI, Macluskey M, Pavitt S, Sloan P, Worthington HV. Interventions for the treatment of oral and oropharyngeal cancers: surgical treatment. Cochrane Database Syst Rev (9):CD006205. doi:CD006205, 2011.

(17) Flach GB, Tenhagen M, de Bree R, Brakenhoff RH, van der Waal I, Bloemena E, Kuik DJ, Castelijns JA, Leemans CR. Outcome of patients with early stage oral cancer managed by an observation strategy towards the N0 neck using ultrasound guided fine needle aspiration cytology: No survival difference as compared to elective neck dissection. Oral Oncol 49:157-164, 2013.

(18) Petrelli F, Coinu A, Riboldi V, Borgonovo K, Ghilardi M, Cabiddu M, Lonati V, Sarti E, Barni S. Concomitant platinum-based chemotherapy or cetuximab with radiotherapy for locally advanced head and neck cancer: A systematic review and meta-analysis of published studies. Oral Oncol 50:1041-1048, 2014.

(19) Alho OP, Kantola S, Pirkola U, Laara E, Jokinen K, Pukkala E. Cancer of the mobile tongue in Finland--increasing incidence, but improved survival. Acta Oncol 38:1021-1024, 1999.

(20) Al-Rajhi N, Khafaga Y, El-Husseiny J, Saleem M, Mourad W, Al-Otieschan A, Al-Amro A. Early stage carcinoma of oral tongue: prognostic factors for local control and survival. Oral Oncol 36:508-514, 2000.

(21) Makitie AA, Koivunen P, Keski-Santti H, Tornwall J, Pukkila M, Laranne J, Luukkaa M, Vuola J, Joensuu T, Kajanti M, Grenman R. Oral tongue carcinoma and its treatment in Finland. Eur Arch Otorhinolaryngol 264:263-267, 2007.

(22) Bonnardot L, Bardet E, Steichen O, Cassagnau E, Piot B, Salam AP, Campion L, Ferron C, Beauvillain de Montreuil C, Malard O. Prognostic factors for T1-T2 squamous cell carcinomas of the mobile tongue: A retrospective cohort study. Head Neck 33:928-934, 2011.

(23) Ganly I, Patel S, Shah J. Early stage squamous cell cancer of the oral tongue--clinicopathologic features affecting outcome. Cancer 118:101-111, 2012.

(24) Rodrigues PC, Miguel MC, Bagordakis E, Fonseca FP, de Aquino SN, Santos-Silva AR, Lopes MA, Graner E, Salo T, Kowalski LP, Coletta RD. Clinicopathological prognostic factors of oral tongue squamous cell carcinoma: a retrospective study of 202 cases. Int J Oral Maxillofac Surg 43:795-801, 2014.

(25) Sano D, Myers JN. Metastasis of squamous cell carcinoma of the oral tongue. Cancer Metastasis Rev 26:645-662, 2007.

(26) Bello IO, Soini Y, Salo T. Prognostic evaluation of oral tongue cancer: means, markers and perspectives (I). Oral Oncol 46:630-635, 2010.

(27) Bello IO, Soini Y, Salo T. Prognostic evaluation of oral tongue cancer: means, markers and perspectives (II). Oral Oncol 46:636-643, 2010.

(28) Ferlito A, Buckley JG, Shaha AR, Rinaldo A. Contemporary important considerations in diagnosis and treatment of head and neck cancer. Acta Otolaryngol (Stockh) 122:115-120, 2002.

(29) Ferlito A, Rinaldo A, Devaney KO, MacLennan K, Myers JN, Petruzzelli GJ, Shaha AR, Genden EM, Johnson JT, de Carvalho MB, Myers EN. Prognostic significance of microscopic and macroscopic extracapsular spread from metastatic tumor in the cervical lymph nodes. Oral Oncol 38:747-751, 2002.

(30) Ferlito A, Shaha AR, Rinaldo A. The incidence of lymph node micrometastases in patients pathologically staged N0 in cancer of oral cavity and oropharynx. Oral Oncol 38:3-5, 2002.

(31) Arduino PG, Carrozzo M, Chiecchio A, Broccoletti R, Tirone F, Borra E, Bertolusso G, Gandolfo S. Clinical and histopathologic independent prognostic factors in oral squamous cell carcinoma: a retrospective study of 334 cases. J Oral Maxillofac Surg 66:1570-1579, 2008.

(32) Shah JP. Cervical lymph node metastases--diagnostic, therapeutic, and prognostic implications. Oncology (Williston Park) 4:61-9; discussion 72, 76, 1990.

(33) Howell GM, Grandis JR. Molecular mediators of metastasis in head and neck squamous cell carcinoma. Head Neck 27:710-717, 2005.

(34) Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. Oral Oncol 42:229-239, 2006.

(35) Keski-Santti H, Atula T, Tikka J, Hollmen J, Makitie AA, Leivo I. Predictive value of histopathologic parameters in early squamous cell carcinoma of oral tongue. Oral Oncol 43:1007-1013, 2007.

(36) Mehanna H, West CM, Nutting C, Paleri V. Head and neck cancer--Part 2: Treatment and prognostic factors. BMJ 341:c4690, 2010.

(37) Shim SJ, Cha J, Koom WS, Kim GE, Lee CG, Choi EC, Keum KC. Clinical outcomes for T1-2N0-1 oral tongue cancer patients underwent surgery with and without postoperative radiotherapy. Radiat Oncol 5:43-717X-5-43, 2010.

(38) Tai SK, Li WY, Yang MH, Chu PY, Wang YF, Chang PM. Perineural invasion as a major determinant for the aggressiveness associated with increased tumor thickness in t1-2 oral tongue and buccal squamous cell carcinoma. Ann Surg Oncol 20:3568-3574, 2013.

(39) Piazza C, Montalto N, Paderno A, Taglietti V, Nicolai P. Is it time to incorporate 'depth of infiltration' in the T staging of oral tongue and floor of mouth cancer? Curr Opin Otolaryngol Head Neck Surg 22:81-89, 2014.

(40) Zhang T, Lubek JE, Salama A, Dyalram D, Liu X, Ord RA. Treatment of cT1N0M0 tongue cancer: outcome and prognostic parameters. J Oral Maxillofac Surg 72:406-414, 2014.

(41) Thiagarajan S, Nair S, Nair D, Chaturvedi P, Kane SV, Agarwal JP, D'Cruz AK. Predictors of prognosis for squamous cell carcinoma of oral tongue. J Surg Oncol 109:639-644, 2014.

(42) Aivazian K, Ebrahimi A, Low TH, Gao K, Clifford A, Shannon K, Clark JR, Gupta R. Perineural invasion in oral squamous cell carcinoma: Quantitative subcategorisation of perineural invasion and prognostication. J Surg Oncol 2014.

(43) Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, Golub LM, Brown DL, Mantyla P. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. Ann Med 38:306-321, 2006.

(44) Johansson N, Ahonen M, Kahari VM. Matrix metalloproteinases in tumor invasion. Cell Mol Life Sci 57:5-15, 2000.

(45) Wahlgren J, Maisi P, Sorsa T, Sutinen M, Tervahartiala T, Pirila E, Teronen O, Hietanen J, Tjaderhane L, Salo T. Expression and induction of collagenases (MMP-8 and -13) in plasma cells associated with bone-destructive lesions. J Pathol 194:217-224, 2001.

(46) Rosenthal EL, Matrisian LM. Matrix metalloproteases in head and neck cancer. Head Neck 28:639-648, 2006.

(47) Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52-67, 2010.

(48) Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25:9-34, 2006.

(49) Martin MD, Matrisian LM. The other side of MMPs: protective roles in tumor progression. Cancer Metastasis Rev 26:717-724, 2007.

(50) Lopez-Otin C, Palavalli LH, Samuels Y. Protective roles of matrix metalloproteinases: from mouse models to human cancer. Cell Cycle 8:3657-3662, 2009.

(51) Korpi JT, Kervinen V, Maklin H, Vaananen A, Lahtinen M, Laara E, Ristimaki A, Thomas G, Ylipalosaari M, Astrom P, Lopez-Otin C, Sorsa T, Kantola S, Pirila E, Salo T. Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer. Br J Cancer 98:766-775, 2008.

(52) Gutierrez-Fernandez A, Fueyo A, Folgueras AR, Garabaya C, Pennington CJ, Pilgrim S, Edwards DR, Holliday DL, Jones JL, Span PN, Sweep FC, Puente XS, Lopez-Otin C. Matrix metalloproteinase-8 functions as a metastasis suppressor through modulation of tumor cell adhesion and invasion. Cancer Res 68:2755-2763, 2008.

(53) Kusukawa J, Sasaguri Y, Morimatsu M, Kameyama T. Expression of matrix metalloproteinase-3 in stage I and II squamous cell carcinoma of the oral cavity. J Oral Maxillofac Surg 53:530-534, 1995.

(54) Kurahara S, Shinohara M, Ikebe T, Nakamura S, Beppu M, Hiraki A, Takeuchi H, Shirasuna K. Expression of MMPS, MT-MMP, and TIMPs in squamous cell carcinoma of the oral cavity: correlations with tumor invasion and metastasis. Head Neck 21:627-638, 1999.

(55) Yoshizaki T, Maruyama Y, Sato H, Furukawa M. Expression of tissue inhibitor of matrix metalloproteinase-2 correlates with activation of matrix metalloproteinase-2 and predicts poor prognosis in tongue squamous cell carcinoma. Int J Cancer 95:44-50, 2001.

(56) Culhaci N, Metin K, Copcu E, Dikicioglu E. Elevated expression of MMP-13 and TIMP-1 in head and neck squamous cell carcinomas may reflect increased tumor invasiveness. BMC Cancer 4:42, 2004.

(57) Luukkaa M, Vihinen P, Kronqvist P, Vahlberg T, Pyrhonen S, Kahari VM, Grenman R. Association between high collagenase-3 expression levels and poor prognosis in patients with head and neck cancer. Head Neck 28:225-234, 2006.

(58) de Vicente JC, Lequerica-Fernandez P, Santamaria J, Fresno MF. Expression of MMP-7 and MT1-MMP in oral squamous cell carcinoma as predictive indicator for tumor invasion and prognosis. J Oral Pathol Med 36:415-424, 2007.

(59) Weber A, Hengge UR, Stricker I, Tischoff I, Markwart A, Anhalt K, Dietz A, Wittekind C, Tannapfel A. Protein microarrays for the detection of biomarkers in head and neck squamous cell carcinomas. Hum Pathol 38:228-238, 2007.

(60) Ioannou S, Voulgarelis M. Toll-like receptors, tissue injury, and tumourigenesis. Mediators Inflamm 2010:10.1155/2010/581837. Epub 2010 Sep 14, 2010.

(61) Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Arch Pharm Res 35:1297-1316, 2012.

(62) Pradere JP, Dapito DH, Schwabe RF. The Yin and Yang of Toll-like receptors in cancer. Oncogene 2013.

(63) Yu L, Wang L, Chen S. Dual character of Toll-like receptor signaling: pro-tumorigenic effects and anti-tumor functions. Biochim Biophys Acta 1835:144-154, 2013.

(64) Yang H, Wang B, Yan J, Wang T, Zhou XN, Wen HY, Zhu XM. Toll-Like Receptor 2 Promotes Invasion by SGC-7901 Human Gastric Carcinoma Cells and Is Associated with Gastric Carcinoma Metastasis. Ann Clin Lab Sci 44:158-166, 2014.

(65) Ilmarinen T, Hagstrom J, Haglund C, Auvinen E, Leivo I, Pitkaranta A, Aaltonen LM. Low expression of nuclear Toll-like receptor 4 in laryngeal papillomas transforming into squamous cell carcinoma. Otolaryngol Head Neck Surg 151:785-790, 2014.

(66) He Z, Huang X, Ni Y, Shi P, Wang Z, Han W, Hu Q. Functional toll-like receptor 3 expressed by oral squamous cell carcinoma induced cell apoptosis and decreased migration. Oral Surg Oral Med Oral Pathol Oral Radiol 118:92-100, 2014.

(67) Ahmed Haji Omar A, Korvala J, Haglund C, Virolainen S, Hayry V, Atula T, Kontio R, Rihtniemi J, Pihakari A, Sorsa T, Hagstrom J, Salo T. Toll-like receptors -4 and -5 in oral and cutaneous squamous cell carcinomas. J Oral Pathol Med 2014.

(68) Grimm M, Munz A, Exarchou A, Polligkeit J, Reinert S. Immunohistochemical detection of Helicobacter pylori without association of TLR5 expression in oral squamous cell carcinoma. J Oral Pathol Med 43:35-44, 2014.

(69) Kauppila JH, Korvala J, Siirila K, Manni M, Makinen LK, Hagstrom J, Atula T, Haglund C, Selander KS, Saarnio J, Karttunen TJ, Lehenkari PP, Salo T. Toll-like receptor 9 mediates invasion and predicts prognosis in squamous cell carcinoma of the mobile tongue. J Oral Pathol Med 2014.

(70) Kauppila JH, Mattila AE, Karttunen TJ, Salo T. Toll-like receptor 5 (TLR5) expression is a novel predictive marker for recurrence and survival in squamous cell carcinoma of the tongue. Br J Cancer 108:638-643, 2013.

(71) Chuang HC, Huang CC, Chien CY, Chuang JH. Toll-like receptor 3-mediated tumor invasion in head and neck cancer. Oral Oncol 48:226-232, 2012.

(72) Luo Q, Hu S, Yan M, Sun Z, Chen W, Chen F. Activation of Toll-like receptor 3 induces apoptosis of oral squamous carcinoma cells in vitro and in vivo. Int J Biochem Cell Biol 44:1266-1275, 2012.

(73) Min R, Zun Z, Siyi L, Wenjun Y, Lizheng W, Chenping Z. Increased expression of Toll-like receptor-9 has close relation with tumour cell proliferation in oral squamous cell carcinoma. Arch Oral Biol 56:877-884, 2011.

(74) Rydberg C, Mansson A, Uddman R, Riesbeck K, Cardell LO. Toll-like receptor agonists induce inflammation and cell death in a model of head and neck squamous cell carcinomas. Immunology 128:e600-11, 2009.

(75) Szczepanski MJ, Czystowska M, Szajnik M, Harasymczuk M, Boyiadzis M, Kruk-Zagajewska A, Szyfter W, Zeromski J, Whiteside TL. Triggering of Toll-like receptor 4 expressed on human head and neck squamous cell carcinoma promotes tumor development and protects the tumor from immune attack. Cancer Res 69:3105-3113, 2009.

(76) Pries R, Hogrefe L, Xie L, Frenzel H, Brocks C, Ditz C, Wollenberg B. Induction of c-Myc-dependent cell proliferation through toll-like receptor 3 in head and neck cancer. Int J Mol Med 21:209-215, 2008.

(77) Szczepanski M, Stelmachowska M, Stryczynski L, Golusinski W, Samara H, Mozer-Lisewska I, Zeromski J. Assessment of expression of toll-like receptors 2, 3 and 4 in laryngeal carcinoma. Eur Arch Otorhinolaryngol 264:525-530, 2007.

(78) Deschler DG DT. 3rd ed. ed.: Alexandria (VA): American Academy of Head and Neck Surgery.; 2008.

(79) Li R, Koch WM, Fakhry C, Gourin CG. Distinct epidemiologic characteristics of oral tongue cancer patients. Otolaryngol Head Neck Surg 148:792-796, 2013.

(80) Mork J, Lie AK, Glattre E, Hallmans G, Jellum E, Koskela P, Moller B, Pukkala E, Schiller JT, Youngman L, Lehtinen M, Dillner J. Human papillomavirus infection as a risk factor for squamous-cell carcinoma of the head and neck. N Engl J Med 344:1125-1131, 2001.

(81) Ang K, Weber R, Vokes E, Trotti A, Chapman D, Cooper J, Forastiere A, Fu KK, Hammond E, Khuri F, Leveque FS, Leupold N, Eisbruch A, Mack C, Mukherji S, Pajak TF, Ridge JA, Radiation Therapy Oncology Group. Radiation Therapy Oncology Group. Research Plan 2002-2006. Head and Neck Cancer Committee. Int J Radiat Oncol Biol Phys 51:39-43, 2001.

(82) Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsague X, Laporte L, Bosch FX, de Sanjose S, Trottier H. HPV DNA, E6/E7 mRNA, and p16(INK4a) detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol 15:1319-1331, 2014.

(83) Conway DI, Hashibe M, Boffetta P, INHANCE consortium, Wunsch-Filho V, Muscat J, La Vecchia C, Winn DM. Enhancing epidemiologic research on head and neck cancer: INHANCE - The international head and neck cancer epidemiology consortium. Oral Oncol 45:743-746, 2009.

(84) Stoeckli SJ, Broglie MA. Sentinel node biopsy for early oral carcinoma. Curr Opin Otolaryngol Head Neck Surg 20:103-108, 2012.

(85) Atula T, Hunter KD, Cooper LA, Shoaib T, Ross GL, Soutar DS. Micrometastases and isolated tumour cells in sentinel lymph nodes in oral and oropharyngeal squamous cell carcinoma. Eur J Surg Oncol 35:532-538, 2009.

(86) Den Toom IJ, Heuveling DA, Flach GB, van Weert S, Karagozoglu KH, van Schie A, Bloemena E, Leemans CR, de Bree R. Sentinel node biopsy for early-stage oral cavity cancer: the VU University Medical Center experience. Head Neck 2014.

(87) Sobin LH, Gospodarowicz MK, Wittekind C. TNM Classification of Malignant Tumours. 7th ed. New York: Wiley-Blackwell; 2009.

(88) Shah JP, Gil Z. Current concepts in management of oral cancer--surgery. Oral Oncol 45:394-401, 2009.

(89) Chaturvedi P, Datta S, Arya S, Rangarajan V, Kane SV, Nair D, Nair S, Chaukar DA, Pai PS, Pantvaidya G, Deshmukh AD, Agrawal A, D'Cruz AK. Prospective study of ultrasound-guided fine-needle aspiration cytology and sentinel node biopsy in the staging of clinically negative T1 and T2 oral cancer. Head Neck 2014.

(90) Psychogios G, Mantsopoulos K, Bohr C, Koch M, Zenk J, Iro H. Incidence of occult cervical metastasis in head and neck carcinomas: development over time. J Surg Oncol 107:384-387, 2013.

(91) Keski-Santti H, Atula T, Tornwall J, Koivunen P, Makitie A. Elective neck treatment versus observation in patients with T1/T2 N0 squamous cell carcinoma of oral tongue. Oral Oncol 42:96-101, 2006.

(92) Furness S, Glenny AM, Worthington HV, Pavitt S, Oliver R, Clarkson JE, Macluskey M, Chan KK, Conway DI. Interventions for the treatment of oral cavity and oropharyngeal cancer: chemotherapy. Cochrane Database Syst Rev (4):CD006386. doi:CD006386, 2011.

(93) Amit M, Yen TC, Liao CT, Chaturvedi P, Agarwal JP, Kowalski LP, Ebrahimi A, Clark JR, Kreppel M, Zoller J, Fridman E, Bolzoni VA, Shah JP, Binenbaum Y, Patel SG, Gil Z, The International Consortium for Outcome Research (ICOR) in Head and Neck Cancer. Improvement in survival of patients with oral cavity squamous cell carcinoma: An International Collaborative Study. Cancer 2013.

(94) Bell RB, Kademani D, Homer L, Dierks EJ, Potter BE. Tongue cancer: Is there a difference in survival compared with other subsites in the oral cavity? J Oral Maxillofac Surg 65:229-236, 2007.

(95) Rusthoven K, Ballonoff A, Raben D, Chen C. Poor prognosis in patients with stage I and II oral tongue squamous cell carcinoma. Cancer 112:345-351, 2008.

(96) Rusthoven KE, Raben D, Song JI, Kane M, Altoos TA, Chen C. Survival and patterns of relapse in patients with oral tongue cancer. J Oral Maxillofac Surg 68:584-589, 2010.

(97) Davidson BJ, Root WA, Trock BJ. Age and survival from squamous cell carcinoma of the oral tongue. Head Neck 23:273-279, 2001.

(98) Annertz K, Anderson H, Biorklund A, Moller T, Kantola S, Mork J, Olsen JH, Wennerberg J. Incidence and survival of squamous cell carcinoma of the tongue in Scandinavia, with special reference to young adults. Int J Cancer 101:95-99, 2002.

(99) Kantola S, Parikka M, Jokinen K, Hyrynkangs K, Soini Y, Alho OP, Salo T. Prognostic factors in tongue cancer - relative importance of demographic, clinical and histopathological factors. Br J Cancer 83:614-619, 2000.

(100) Rosenquist K. Risk factors in oral and oropharyngeal squamous cell carcinoma: a population-based case-control study in southern Sweden. Swed Dent J Suppl (179):1-66, 2005.

(101) Amit M, Yen TC, Liao CT, Binenbaum Y, Chaturvedi P, Agarwal JP, Kowalski LP, Ebrahimi A, Clark JR, Cernea CR, Brandao SJ, Kreppel M, Zoller J, Fliss D, Bachar G, Shpitzer T, Bolzoni VA, Patel PR, Jonnalagadda S, Robbins KT, Shah JP, Patel SG, Gil Z, International Consortium for Outcome Research (ICOR) in Head and Neck Cancer. Clinical nodal stage is a significant predictor of outcome in patients with oral cavity squamous cell carcinoma and pathologically negative neck metastases: results of the international consortium for outcome research. Ann Surg Oncol 20:3575-3581, 2013.

(102) Sayed SI, Sharma S, Rane P, Vaishampayan S, Talole S, Chaturvedi P, Chaukar D, Deshmukh A, Agarwal JP, D'cruz AK. Can metastatic lymph node ratio (LNR) predict survival in oral cavity cancer patients? J Surg Oncol 108:256-263, 2013.

(103) Montero PH, Yu C, Palmer FL, Patel PD, Ganly I, Shah JP, Shaha AR, Boyle JO, Kraus DH, Singh B, Wong RJ, Morris LG, Kattan MW, Patel SG. Nomograms for preoperative prediction of prognosis in patients with oral cavity squamous cell carcinoma. Cancer 120:214-221, 2014.

(104) Shibuya Y, Ohtsuki Y, Hirai C, Hasegawa T, Akashi M, Shigeta T, Minamikawa T, Komori T. Oral squamous cell carcinoma with microscopic extracapsular spread in the cervical lymph nodes. Int J Oral Maxillofac Surg 2013.

(105) Sutton DN, Brown JS, Rogers SN, Vaughan ED, Woolgar JA. The prognostic implications of the surgical margin in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 32:30-34, 2003.

(106) Rogers SN, Brown JS, Woolgar JA, Lowe D, Magennis P, Shaw RJ, Sutton D, Errington D, Vaughan D. Survival following primary surgery for oral cancer. Oral Oncol 45:201-211, 2009.

(107) Nason RW, Binahmed A, Pathak KA, Abdoh AA, Sandor GK. What is the adequate margin of surgical resection in oral cancer? Oral Surg Oral Med Oral Pathol Oral Radiol Endod 107:625-629, 2009.

(108) Brandwein-Gensler M, Teixeira MS, Lewis CM, Lee B, Rolnitzky L, Hille JJ, Genden E, Urken ML, Wang BY. Oral squamous cell carcinoma: histologic risk assessment, but not margin status, is strongly predictive of local disease-free and overall survival. Am J Surg Pathol 29:167-178, 2005.

(109) Kurita H, Nakanishi Y, Nishizawa R, Xiao T, Kamata T, Koike T, Kobayashi H. Impact of different surgical margin conditions on local recurrence of oral squamous cell carcinoma. Oral Oncol 46:814-817, 2010.

(110) Kademani D, Bell RB, Bagheri S, Holmgren E, Dierks E, Potter B, Homer L. Prognostic factors in intraoral squamous cell carcinoma: the influence of histologic grade. J Oral Maxillofac Surg 63:1599-1605, 2005.

(111) Thomas B, Stedman M, Davies L. Grade as a prognostic factor in oral squamous cell carcinoma: a population-based analysis of the data. Laryngoscope 124:688-694, 2014.

(112) Almangush A, Salo T, Hagstrom J, Leivo I. Tumour budding in head and neck squamous cell carcinoma - a systematic review. Histopathology 65:587-594, 2014.

(113) Almangush A, Bello IO, Keski-Santti H, Makinen LK, Kauppila JH, Pukkila M, Hagstrom J, Laranne J, Tommola S, Nieminen O, Soini Y, Kosma VM, Koivunen P, Grenman R, Leivo I, Salo T. Depth of invasion, tumor budding and worst pattern of invasion classify patients with early stage oral tongue cancer into low- and high-risk categories of mortality. Head Neck 2013.

(114) Almangush A, Coletta RD, Bello IO, Bitu C, Keski-Santti H, Makinen LK, Kauppila JH, Pukkila M, Hagstrom J, Laranne J, Tommola S, Soini Y, Kosma VM, Koivunen P, Kowalski LP, Nieminen P, Grenman R, Leivo I, Salo T. A simple novel prognostic model for early stage oral tongue cancer. Int J Oral Maxillofac Surg 2014.

(115) Xie N, Wang C, Liu X, Li R, Hou J, Chen X, Huang H. Tumor budding correlates with occult cervical lymph node metastasis and poor prognosis in clinical early-stage tongue squamous cell carcinoma. J Oral Pathol Med 2014.

(116) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 144:646-674, 2011.

(117) Nyberg P, Salo T, Kalluri R. Tumor microenvironment and angiogenesis. Front Biosci 13:6537-6553, 2008.

(118) Salo T, Vered M, Bello IO, Nyberg P, Bitu CC, Zlotogorski Hurvitz A, Dayan D. Insights into the role of components of the tumor microenvironment in oral carcinoma call for new therapeutic approaches. Exp Cell Res 325:58-64, 2014.

(119) Han MW, Lee JC, Kim YM, Cha HJ, Roh JL, Choi SH, Nam SY, Cho KJ, Kim SW, Kim SY. Epithelial-Mesenchymal Transition: Clinical Implications for Nodal Metastasis and Prognosis of Tongue Cancer. Otolaryngol Head Neck Surg 2014.

(120) Seethala RR. Histologic grading and prognostic biomarkers in salivary gland carcinomas. Adv Anat Pathol 18:29-45, 2011.

(121) Larsen SR, Bjorndal K, Godballe C, Krogdahl A. Prognostic significance of Ki-67 in salivary gland carcinomas. J Oral Pathol Med 41:598-602, 2012.

(122) Mirghani H, Amen F, Moreau F, Guigay J, Ferchiou M, Melkane AE, Hartl DM, Lacau St Guily J. Human papilloma virus testing in oropharyngeal squamous cell carcinoma: what the clinician should know. Oral Oncol 50:1-9, 2014.

(123) Chung CH, Gillison ML. Human papillomavirus in head and neck cancer: its role in pathogenesis and clinical implications. Clin Cancer Res 15:6758-6762, 2009.

(124) Yokota T. Is biomarker research advancing in the era of personalized medicine for head and neck cancer? Int J Clin Oncol 19:211-219, 2014.

(125) Bockelman C, Hagstrom J, Makinen LK, Keski-Santti H, Hayry V, Lundin J, Atula T, Ristimaki A, Haglund C. High CIP2A immunoreactivity is an independent prognostic indicator in early-stage tongue cancer. Br J Cancer 104:1890-1895, 2011.

(126) Hayry V, Makinen LK, Atula T, Sariola H, Makitie A, Leivo I, Keski-Santti H, Lundin J, Haglund C, Hagstrom J. Bmi-1 expression predicts prognosis in squamous cell carcinoma of the tongue. Br J Cancer 102:892-897, 2010.

(127) Hwa JS, Kwon OJ, Park JJ, Woo SH, Kim JP, Ko GH, Seo JH, Kim RB. The prognostic value of immunohistochemical markers for oral tongue squamous cell carcinoma. Eur Arch Otorhinolaryngol 2014.

(128) Takes RP, Rinaldo A, Rodrigo JP, Devaney KO, Fagan JJ, Ferlito A. Can biomarkers play a role in the decision about treatment of the clinically negative neck in patients with head and neck cancer? Head Neck 30:525-538, 2008.

(129) Wikner J, Grobe A, Pantel K, Riethdorf S. Squamous cell carcinoma of the oral cavity and circulating tumour cells. World J Clin Oncol 5:114-124, 2014.

(130) Diaz LA,Jr, Bardelli A. Liquid biopsies: genotyping circulating tumor DNA. J Clin Oncol 32:579-586, 2014.

(131) Peters DL, Pretorius PJ. Origin, translocation and destination of extracellular occurring DNA--a new paradigm in genetic behaviour. Clin Chim Acta 412:806-811, 2011.

(132) Jahr S, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. Cancer Res 61:1659-1665, 2001.

(133) Hua H, Li M, Luo T, Yin Y, Jiang Y. Matrix metalloproteinases in tumorigenesis: an evolving paradigm. Cell Mol Life Sci 68:3853-3868, 2011.

(134) Bourboulia D, Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20:161-168, 2010.

(135) Lynch CC, Matrisian LM. Matrix metalloproteinases in tumor-host cell communication. Differentiation 70:561-573, 2002.

(136) Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev 14:2123-2133, 2000.

(137) Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem 253:269-285, 2003.

(138) Folgueras AR, Pendas AM, Sanchez LM, Lopez-Otin C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. Int J Dev Biol 48:411-424, 2004.

(139) Vihinen P, Kahari VM. Matrix metalloproteinases in cancer: prognostic markers and therapeutic targets. Int J Cancer 99:157-166, 2002.

(140) Montel V, Kleeman J, Agarwal D, Spinella D, Kawai K, Tarin D. Altered metastatic behavior of human breast cancer cells after experimental manipulation of matrix metalloproteinase 8 gene expression. Cancer Res 64:1687-1694, 2004.

(141) Perez-Sayans Garcia M, Suarez-Penaranda JM, Gayoso-Diz P, Barros-Angueira F, Gandara-Rey JM, Garcia-Garcia A. Tissue inhibitor of metalloproteinases in oral squamous cell carcinomas - a therapeutic target? Cancer Lett 323:11-19, 2012.

(142) Ala-aho r, Kahari VM. Collagenases in cancer. Biochimie 87:273-286, 2005.

(143) Lopez-Otin C, Matrisian LM. Emerging roles of proteases in tumour suppression. Nat Rev Cancer 7:800-808, 2007.

(144) de Amorim RF, da Silveira EJ, Queiroz LM, Galvao HC, de Souza LB, de Almeida Freitas R. Matrilysins may not predict the metastatic potential in squamous cell carcinoma of the tongue. Acta Odontol Scand 68:228-231, 2010.

(145) Kahari VM, Saarialho-Kere U. Matrix metalloproteinases and their inhibitors in tumour growth and invasion. Ann Med 31:34-45, 1999.

(146) Yamamoto H, Itoh F, Iku S, Adachi Y, Fukushima H, Sasaki S, Mukaiya M, Hirata K, Imai K. Expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human pancreatic adenocarcinomas: clinicopathologic and prognostic significance of matrilysin expression. J Clin Oncol 19:1118-1127, 2001.

(147) Wang WS, Chen PM, Wang HS, Liang WY, Su Y. Matrix metalloproteinase-7 increases resistance to Fas-mediated apoptosis and is a poor prognostic factor of patients with colorectal carcinoma. Carcinogenesis 27:1113-1120, 2006.

(148) Fang YJ, Pan ZZ, Li LR, Lu ZH, Zhang LY, Wan DS. MMP7 expression regulated by endocrine therapy in ERbeta-positive colon cancer cells. J Exp Clin Cancer Res 28:132, 2009.

(149) Yang B, Su K, Gao J, Rao Z. Expression and prognostic value of matrix metalloproteinase-7 in colorectal cancer. Asian Pac J Cancer Prev 13:1049-1052, 2012.

(150) Fanelli MF, Chinen LT, Begnami MD, Costa WL,Jr, Fregnami JH, Soares FA, Montagnini AL. The influence of transforming growth factor-alpha, cyclooxygenase-2, matrix metalloproteinase (MMP)-7, MMP-9 and CXCR4 proteins involved in epithelial-mesenchymal transition on overall survival of patients with gastric cancer. Histopathology 61:153-161, 2012.

(151) Hirashita T, Iwashita Y, Ohta M, Komori Y, Eguchi H, Yada K, Kitano S. Expression of matrix metalloproteinase-7 is an unfavorable prognostic factor in intrahepatic cholangiocarcinoma. J Gastrointest Surg 16:842-848, 2012.

(152) Ii M, Yamamoto H, Adachi Y, Maruyama Y, Shinomura Y. Role of matrix metalloproteinase-7 (matrilysin) in human cancer invasion, apoptosis, growth, and angiogenesis. Exp Biol Med (Maywood) 231:20-27, 2006.

(153) English WR, Holtz B, Vogt G, Knauper V, Murphy G. Characterization of the role of the "MT-loop": an eight-amino acid insertion specific to progelatinase A (MMP2) activating membrane-type matrix metalloproteinases. J Biol Chem 276:42018-42026, 2001.

(154) Sohail A, Sun Q, Zhao H, Bernardo MM, Cho JA, Fridman R. MT4-(MMP17) and MT6-MMP (MMP25), A unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer. Cancer Metastasis Rev 27:289-302, 2008.

(155) Kuula H, Salo T, Pirila E, Hagstrom J, Luomanen M, Gutierrez-Fernandez A, Romanos GE, Sorsa T. Human beta-defensin-1 and -2 and matrix metalloproteinase-25 and -26 expression in chronic and aggressive periodontitis and in peri-implantitis. Arch Oral Biol 53:175-186, 2008.

(156) Sun Q, Weber CR, Sohail A, Bernardo MM, Toth M, Zhao H, Turner JR, Fridman R. MMP25 (MT6-MMP) is highly expressed in human colon cancer, promotes tumor growth, and exhibits unique biochemical properties. J Biol Chem 282:21998-22010, 2007.

(157) Chuang HC, Su CY, Huang HY, Huang CC, Chien CY, Du YY, Chuang JH. Active matrix metalloproteinase-7 is associated with invasion in buccal squamous cell carcinoma. Mod Pathol 21:1444-1450, 2008.

(158) Vincent-Chong VK, Salahshourifar I, Karen-Ng LP, Siow MY, Kallarakkal TG, Ramanathan A, Yang YH, Khor GH, Rahman ZA, Ismail SM, Prepageran N, Wan Mustafa WM, Abraham MT, Tay KK, Cheong SC, Zain RB. Overexpression of MMP13 Is Associated with Clinical Outcomes and Poor Prognosis in Oral Squamous Cell Carcinoma. ScientificWorldJournal 2014:897523, 2014.

(159) Ahmed Haji Omar A, Haglund C, Virolainen S, Hayry V, Atula T, Kontio R, Salo T, Sorsa T, Hagstrom J. MMP-7, MMP-8, and MMP-9 in oral and cutaneous squamous cell carcinomas. Oral Surg Oral Med Oral Pathol Oral Radiol 119:459-467, 2015.

(160) Hong SD, Hong SP, Lee JI, Lim CY. Expression of matrix metalloproteinase-2 and -9 in oral squamous cell carcinomas with regard to the metastatic potential. Oral Oncol 36:207-213, 2000.

(161) Sutinen M, Kainulainen T, Hurskainen T, Vesterlund E, Alexander JP, Overall CM, Sorsa T, Salo T. Expression of matrix metalloproteinases (MMP-1 and -2) and their inhibitors (TIMP-1, -2 and -3) in oral lichen planus, dysplasia, squamous cell carcinoma and lymph node metastasis. Br J Cancer 77:2239-2245, 1998.

(162) Katayama A, Bandoh N, Kishibe K, Takahara M, Ogino T, Nonaka S, Harabuchi Y. Expressions of matrix metalloproteinases in early-stage oral squamous cell carcinoma as predictive indicators for tumor metastases and prognosis. Clin Cancer Res 10:634-640, 2004.

(163) Barros SS, Henriques AC, Pereira KM, de Medeiros AM, Galvao HC, Freitas Rde A. Immunohistochemical expression of matrix metalloproteinases in squamous cell carcinoma of the tongue and lower lip. Arch Oral Biol 56:752-760, 2011.

(164) Aparna M, Rao L, Kunhikatta V, Radhakrishnan R. The role of MMP-2 and MMP-9 as prognostic markers in the early stages of tongue squamous cell carcinoma. J Oral Pathol Med 2014.

(165) O-Charoenrat P, Rhys-Evans PH, Eccles SA. Expression of matrix metalloproteinases and their inhibitors correlates with invasion and metastasis in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 127:813-820, 2001.

(166) Franchi A, Santucci M, Masini E, Sardi I, Paglierani M, Gallo O. Expression of matrix metalloproteinase 1, matrix metalloproteinase 2, and matrix metalloproteinase 9 in carcinoma of the head and neck. Cancer 95:1902-1910, 2002.

(167) Kawata R, Shimada T, Maruyama S, Hisa Y, Takenaka H, Murakami Y. Enhanced production of matrix metalloproteinase-2 in human head and neck carcinomas is correlated with lymph node metastasis. Acta Otolaryngol (Stockh) 122:101-106, 2002.

(168) Moilanen M, Pirila E, Grenman R, Sorsa T, Salo T. Expression and regulation of collagenase-2 (MMP-8) in head and neck squamous cell carcinomas. J Pathol 197:72-81, 2002.

(169) Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T. Expression of matrix metalloproteinase-9 in head and neck squamous cell carcinoma: a potential marker for prognosis. Clin Cancer Res 10:3110-3116, 2004.

(170) Ruokolainen H, Paakko P, Turpeenniemi-Hujanen T. Tissue and circulating immunoreactive protein for MMP-2 and TIMP-2 in head and neck squamous cell carcinoma--tissue immunoreactivity predicts aggressive clinical course. Mod Pathol 19:208-217, 2006.

(171) Bodnar M, Szylberg L, Kazmierczak W, Marszalek A. Tumor progression driven by pathways activating matrix metalloproteinases and their inhibitors. J Oral Pathol Med 2014.

(172) Corte MD, Gonzalez LO, Corte MG, Quintela I, Pidal I, Bongera M, Vizoso F. Collagenase-3 (MMP-13) expression in cutaneous malignant melanoma. Int J Biol Markers 20:242-248, 2005.

(173) Zhang B, Cao X, Liu Y, Cao W, Zhang F, Zhang S, Li H, Ning L, Fu L, Niu Y, Niu R, Sun B, Hao X. Tumor-derived matrix metalloproteinase-13 (MMP-13) correlates with poor prognoses of invasive breast cancer. BMC Cancer 8:83, 2008.

(174) Cazorla M, Hernandez L, Nadal A, Balbin M, Lopez JM, Vizoso F, Fernandez PL, Iwata K, Cardesa A, Lopez-Otin C, Campo E. Collagenase-3 expression is associated with advanced local invasion in human squamous cell carcinomas of the larynx. J Pathol 186:144-150, 1998.

(175) Marcos CA, Martinez DA, de Los Toyos JR, Dominguez Iglesias F, Hermsen M, Guervos MA, Pendas JL. The usefulness of new serum tumor markers in head and neck squamous cell carcinoma. Otolaryngol Head Neck Surg 140:375-380, 2009.

(176) Luukkaa H, Klemi P, Hirsimaki P, Vahlberg T, Kivisaari A, Kahari VM, Grenman R. Matrix metalloproteinase (MMP)-1, -9 and -13 as prognostic factors in salivary gland cancer. Acta Otolaryngol 128:482-490, 2008.

(177) Luukkaa H, Klemi P, Hirsimaki P, Vahlberg T, Kivisaari A, Kahari VM, Grenman R. Matrix metalloproteinase (MMP)-7 in salivary gland cancer. Acta Oncol 49:85-90, 2010.

(178) Pradhan-Palikhe P, Vesterinen T, Tarkkanen J, Leivo I, Sorsa T, Salo T, Mattila PS. Plasma level of tissue inhibitor of matrix metalloproteinase-1 but not that of matrix metalloproteinase-8 predicts survival in head and neck squamous cell cancer. Oral Oncol 46:514-518, 2010.

(179) Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 117:979-87; quiz 988, 2006.

(180) Miller LS, Modlin RL. Toll-like receptors in the skin. Semin Immunopathol 29:15-26, 2007.

(181) Siponen M, Kauppila JH, Soini Y, Salo T. TLR4 and TLR9 are induced in oral lichen planus. J Oral Pathol Med 41:741-747, 2012.

(182) Sun Z, Luo Q, Ye D, Chen W, Chen F. Role of toll-like receptor 4 on the immune escape of human oral squamous cell carcinoma and resistance of cisplatin-induced apoptosis. Mol Cancer 11:33-4598-11-33, 2012.

(183) Ng LK, Rich AM, Hussaini HM, Thomson WM, Fisher AL, Horne LS, Seymour GJ. Toll-like receptor 2 is present in the microenvironment of oral squamous cell carcinoma. Br J Cancer 104:460-463, 2011.

(184) Vacchelli E, Eggermont A, Sautes-Fridman C, Galon J, Zitvogel L, Kroemer G, Galluzzi L. Trial Watch: Toll-like receptor agonists for cancer therapy. Oncoimmunology 2:e25238, 2013.

(185) Micali G, Lacarrubba F, Nasca MR, Ferraro S, Schwartz RA. Topical pharmacotherapy for skin cancer: part II. Clinical applications. J Am Acad Dermatol 70:979.e1-12; quiz 9912, 2014.

(186) Sligh JE,Jr. New therapeutic options for actinic keratosis and basal cell carcinoma. Semin Cutan Med Surg 33:S76-80, 2014.

(187) Li TT, Ogino S, Qian ZR. Toll-like receptor signaling in colorectal cancer: Carcinogenesis to cancer therapy. World J Gastroenterol 20:17699-17708, 2014.

(188) Kauppila JH, Selander KS. Toll-Like Receptors in Esophageal Cancer. Front Immunol 5:200, 2014.

(189) Husseinzadeh N, Davenport SM. Role of Toll-like receptors in cervical, endometrial and ovarian cancers: A review. Gynecol Oncol 135:359-363, 2014.

(190) Lu Q, Ding H, Li W. Role of Toll-like receptors in microbiota-associated gastrointestinal cancer metastasis. J Cancer Res Ther 9 Suppl:S142-9, 2013.

(191) Kauppila JH, Takala H, Selander KS, Lehenkari PP, Saarnio J, Karttunen TJ. Increased Toll-like receptor 9 expression indicates adverse prognosis in oesophageal adenocarcinoma. Histopathology 59:643-649, 2011.

(192) Mai CW, Kang YB, Pichika MR. Should a Toll-like receptor 4 (TLR-4) agonist or antagonist be designed to treat cancer? TLR-4: its expression and effects in the ten most common cancers. Onco Targets Ther 6:1573-1587, 2013.

(193) Hagstrom J, Heikkila A, Siironen P, Louhimo J, Heiskanen I, Maenpaa H, Arola J, Haglund C. TLR-4 expression and decrease in chronic inflammation: indicators of aggressive follicular thyroid carcinoma. J Clin Pathol 65:333-338, 2012.

(194) Ruan M, Zhang Z, Li S, Yan M, Liu S, Yang W, Wang L, Zhang C. Activation of Toll-like receptor-9 promotes cellular migration via up-regulating MMP-2 expression in oral squamous cell carcinoma. PLoS One 9:e92748, 2014.

(195) Ahn MY, Kwon SM, Cheong HH, Park JH, Lee J, Min SK, Ahn SG, Yoon JH. Toll-like receptor 7 agonist, imiquimod, inhibits oral squamous carcinoma cells through apoptosis and necrosis. J Oral Pathol Med 41:540-546, 2012.

(196) Park JH, Yoon HE, Jeon DI, Ahn SG, Yoon JH. Activation of TLR2 and TLR5 did not affect tumor progression of an oral squamous cell carcinoma, YD-10B cells. J Oral Pathol Med 39:781-785, 2010.

(197) Marur S, D'Souza G, Westra WH, Forastiere AA. HPV-associated head and neck cancer: a virus-related cancer epidemic. Lancet Oncol 11:781-789, 2010.

(198) Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, Westra WH, Chung CH, Jordan RC, Lu C, Kim H, Axelrod R, Silverman CC, Redmond KP, Gillison ML. Human papillomavirus and survival of patients with oropharyngeal cancer. N Engl J Med 363:24-35, 2010.

(199) Zaravinos A. An updated overview of HPV-associated head and neck carcinomas. Oncotarget 5:3956-3969, 2014.

(200) Friedman JM, Stavas MJ, Cmelak AJ. Clinical and scientific impact of human papillomavirus on head and neck cancer. World J Clin Oncol 5:781-791, 2014.

(201) Lim AM, Do H, Young RJ, Wong SQ, Angel C, Collins M, Takano EA, Corry J, Wiesenfeld D, Kleid S, Sigston E, Lyons B, Fox SB, Rischin D, Dobrovic A, Solomon B. Differential mechanisms of CDKN2A (p16) alteration in oral tongue squamous cell carcinomas and correlation with patient outcome. Int J Cancer 2014.

(202) Deng Z, Hasegawa M, Aoki K, Matayoshi S, Kiyuna A, Yamashita Y, Uehara T, Agena S, Maeda H, Xie M, Suzuki M. A comprehensive evaluation of human papillomavirus positive status and p16INK4a overexpression as a prognostic biomarker in head and neck squamous cell carcinoma. Int J Oncol 45:67-76, 2014.

(203) Garcia-de Marcos JA, Perez-Zafrilla B, Arriaga A, Arroyo-Rodriguez S, Poblet E. Human papillomavirus in carcinomas of the tongue: clinical and prognostic implications. Int J Oral Maxillofac Surg 2013.

(204) Dunkel J, Vaittinen S, Grenman R, Kinnunen I, Irjala H. Prognostic markers in stage I oral cavity squamous cell carcinoma. Laryngoscope 123:2435-2441, 2013.

(205) Duncan LD, Winkler M, Carlson ER, Heidel RE, Kang E, Webb D. P16 Immunohistochemistry can be used to Detect Human Papillomavirus in Oral Cavity Squamous Cell Carcinoma. J Oral Maxillofac Surg 71:1367-1375, 2013.

(206) Ramshankar V, Soundara VT, Shyamsundar V, Ramani P, Krishnamurthy A. Risk stratification of early stage oral tongue cancers based on HPV status and p16 immunoexpression. Asian Pac J Cancer Prev 15:8351-8359, 2014.

(207) Harris SL, Thorne LB, Seaman WT, Hayes DN, Couch ME, Kimple RJ. Association of p16(INK4a) overexpression with improved outcomes in young patients with squamous cell cancers of the oral tongue. Head Neck 33:1622-1627, 2011.

(208) Kallioniemi OP, Wagner U, Kononen J, Sauter G. Tissue microarray technology for high-throughput molecular profiling of cancer. Hum Mol Genet 10:657-662, 2001.

(209) Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Kochli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter G. Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol 159:2249-2256, 2001.

(210) Hanemaaijer R, Sorsa T, Konttinen YT, Ding Y, Sutinen M, Visser H, van Hinsbergh VW, Helaakoski T, Kainulainen T, Ronka H, Tschesche H, Salo T. Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells. Regulation by tumor necrosis factor-alpha and doxycycline. J Biol Chem 272:31504-31509, 1997.

(211) Nurmenniemi S, Sinikumpu T, Alahuhta I, Salo S, Sutinen M, Santala M, Risteli J, Nyberg P, Salo T. A novel organotypic model mimics the tumor microenvironment. Am J Pathol 175:1281-1291, 2009.

(212) Camp RL, Neumeister V, Rimm DL. A decade of tissue microarrays: progress in the discovery and validation of cancer biomarkers. J Clin Oncol 26:5630-5637, 2008.

(213) Szarvas T, Becker M, vom Dorp F, Gethmann C, Totsch M, Bankfalvi A, Schmid KW, Romics I, Rubben H, Ergun S. Matrix metalloproteinase-7 as a marker of metastasis and predictor of poor survival in bladder cancer. Cancer Sci 101:1300-1308, 2010.

(214) Koskensalo S, Mrena J, Wiksten JP, Nordling S, Kokkola A, Hagstrom J, Haglund C. MMP-7 overexpression is an independent prognostic marker in gastric cancer. Tumour Biol 31:149-155, 2010.

(215) Koskensalo S, Louhimo J, Nordling S, Hagstrom J, Haglund C. MMP-7 as a prognostic marker in colorectal cancer. Tumour Biol 32:259-264, 2011.

(216) Lequerica-Fernandez P, Pena I, Villalain L, Rosado P, de Vicente JC. Carcinoma of the parotid gland: developing prognostic indices. Int J Oral Maxillofac Surg 40:821-828, 2011.

(217) Wiegand S, Dunne AA, Muller HH, Mandic R, Barth P, Davis RK, Werner JA. Metaanalysis of the significance of matrix metalloproteinases for lymph node disease in patients with head and neck squamous cell carcinoma. Cancer 104:94-100, 2005.

(218) Balbin M, Fueyo A, Tester AM, Pendas AM, Pitiot AS, Astudillo A, Overall CM, Shapiro SD, Lopez-Otin C. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet 35:252-257, 2003.

(219) Sharma M, Sah P, Sharma SS, Radhakrishnan R. Molecular changes in invasive front of oral cancer. J Oral Maxillofac Pathol 17:240-247, 2013.

(220) Jouhi L, Renkonen S, Atula T, Makitie A, Haglund C, Hagstrom J. Different Toll-Like Receptor Expression Patterns in Progression toward Cancer. Front Immunol 5:638, 2014.

(221) Maksimovic-Ivanic D, Mijatovic S, Harhaji L, Miljkovic D, Dabideen D, Fan Cheng K, Mangano K, Malaponte G, Al-Abed Y, Libra M, Garotta G, Nicoletti F, Stosic-Grujicic S. Anticancer properties of the novel nitric oxide-donating compound (S,R)-3-phenyl-4,5-dihydro-5-isoxazole acetic acid-nitric oxide in vitro and in vivo. Mol Cancer Ther 7:510-520, 2008.