OULU 2018
D 1442

ACTA UNIVERSITATIS OULUENSIS

Elias Sundquist

THE ROLE OF TUMOR MICROENVIRONMENT ON ORAL TONGUE CANCER INVASION AND PROGNOSIS

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE;
MEDICAL RESEARCH CENTER OULU

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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in the Leena Palotie auditorium (101A) of the Faculty of Medicine (Aapistie 5 A), on 9 February 2018, at 12 noon

UNIVERSITY OF OULU, OULU 2018

Acta Univ. Oul. D 1442, 2018

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ISBN 978-952-62-1764-2 (Paperback)
ISBN 978-952-62-1765-9 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2018

Sundquist, Elias, The role of tumor microenvironment on oral tongue cancer invasion and prognosis.
University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu
Acta Univ. Oul. D 1442, 2018
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Abstract

Oral tongue squamous cell carcinoma (OTSCC) is the most common cancer of the oral cavity. The 5-year mortality of OTSCC remains at about 50%. The tumor microenvironment (TME) is now recognized as an important factor in cancer progression and metastasis, as well as a tool for prognostication. The aim of this study was to elucidate the roles of TME hypoxia and soluble factors on cancer cell migration and invasion, and the prognostic value of two extracellular matrix (ECM) molecules: tenascin-C (TNC) and fibronectin (FN).

Hypoxia was studied using oral squamous cell carcinoma cells in migration and invasion assays. Invasion assays were carried out using a 3D-myoma invasion method. Similarly, the effect of soluble factors as well as ECM alterations were studied using the myoma model: the effect of soluble factors was studied by rinsing the myoma discs prior to experiments, and ECM alterations by lyophilizing and rehydrating. ECM was further studied by analyzing the prognostic value of TNC and FN from OTSCC samples.

The effect of hypoxia was shown to be OTSCC cell line dependent: the effect of hypoxia on migration and invasion was increased in aggressive cell lines. Additionally, the response to hypoxia was altered in rinsed tissue. Tissue rinsing media were analyzed and factors affecting cell motility were found. The TME was found to be pivotal for cancer invasion: invasion was impaired in non-neoplastic tissue. Furthermore, changes in the ECM by lyophilization and rehydration led to a change in the invasion mechanism. High expression of stromal TNC and FN were excellent prognosticators in early-stage OTSCC.

In conclusion, the present study highlighted the role of various TME components in cancer cell invasion as well as prognostication in OTSCC. Additionally, this study provided feasible tools for more precise diagnosis of early-stage OTSCC.

Keywords: cancer invasion, fibronectin, hypoxia, immunohistochemistry, myoma, organotypic cell culture, prognosis, squamous cell carcinoma, tenascin-C, tongue cancer, tumor microenvironment

Sundquist, Elias, Kasvaimen mikroympäristön merkitys kielen levyepiteelikarsinooman invaasiossa ja ennusteessa.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu
Acta Univ. Oul. D 1442, 2018
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä

Liikkuvan kielen levyepiteelikarsinooma (OTSCC) on suuontelon yleisin syöpä. Viiden vuoden kuolleisuus OTSCC:an on edelleen noin 50 %. Kasvaimen mikroympäristön (TME) tiedetään nykyään olevan tärkeässä roolissa syövän kehityksessä ja etäpesäkkeiden muodostuksessa, sekä tarjoavan työkaluja ennusteiden laadintaan. Tämän tutkimuksen tarkoituksena oli selvittää TME:n hypoksian ja liukoisten tekijöiden vaikutusta syöpäsolujen liikkumiseen ja invaasioon ympäröivään kudokseen, sekä tutkia kahden solunulkoisen matriksin (ECM) proteiinin, tenaskiini-C:n (TNC) ja fibronektiinin (FN), vaikutusta OTSCC:n ennusteeseen.

Hypoksian vaikutusta tutkittiin käyttäen suun levyepiteelikarsinoomasoluja liikkuvuus- ja invaasiokokeissa. Invaasiokokeissa hyödynnettiin kolmiulotteista ihmisen myoomaan perustuvaa invaasiomallia. Myös liukoisten tekijöiden ja ECM:n muutosten vaikutusten tutkimisessa käytettiin myoomamallia: liukoisten tekijöiden vaikutusta tutkittiin huuhtomalla myoomakiekot ennen niiden käyttämistä, ja ECM:n muutosten vaikutusta kylmäkuivaamalla ja uudelleen nesteyttämällä myoomakiekot. ECM:ia tutkittiin myös analysoimalla TNC:n ja FN:n värjäytyvyyden merkitystä OTSCC:n ennusteessa.

Hypoksian vaikutus osoittautui solulinjariippuvaiseksi: hypoksia lisäsi kielisyöpäsolujen liikkuvuutta ja invaasiota eniten aggressiivisimmilla solulinjoilla. Lisäksi solujen vaste hypoksialle oli erilainen huuhdotussa kudoksessa. Huuhteluliuos analysoitiin ja siitä löydettiin solujen liikkumiseen vaikuttavia tekijöitä. TME:n havaittiin olevan ratkaisevassa roolissa syöpäsolujen invaasiossa: syöpäsolut eivät kyenneet invasoitumaan lainkaan ei-neoplastiseen kudokseen. Lisäksi muutosten ECM:ssä havaittiin johtavan muutoksiin solujen käyttämässä invaasion mekanismissa. Strooman TNC:n ja FN:n värjäytyvyyden todettiin olevan erinomaisia ennustekijöitä aikaisen vaiheen OTSCC:ssa.

Tiivistettynä voidaan todeta, että tämä tutkimus alleviivasi useiden TME:n komponenttien vaikutusta syövän invaasiolle ja ennusteelle OTSCC:ssä. Lisäksi se tarjoaa käyttökelpoiset työkalut (TNC ja FN) tarkemmalle diagnostiikalle aikaisen vaiheen OTSCC:ssä.

Asiasanat: ennuste, fibronektiini, hypoksia, immunohistokemia, kasvaimen mikroympäristö, kielisyöpä, levyepiteelikarsinooma, myooma, organotyyppiset invaasiomallit, syövän invaasio, tenaskiini-C

To people suffering from tongue cancer

Acknowledgements

This study was conducted between 2011 and 2018 at the Faculty of Medicine as part of Cancer and Translational Medicine Research Unit and Medical Research Center Oulu, University of Oulu.

My deepest gratitude goes to my supervisor professor Tuula Salo who's strict yet constructive guidance and encouragement enabled the completion of this research project. Her astonishing insight and enthusiasm in science reaching beyond our specific field, endless ideas as well as energy and perseverance in pursuing them, and the straightforward style of expressing her thoughts are qualities that make her an excellent scientist, teacher and supervisor.

I want to thank the members of my follow-up group: Lauri Eklund, Markus Mäkinen and Sakari Kellokumpu for overseeing the completion of this work.

I am deeply grateful for Timo Paavonen and Outi Monni for reviewing this thesis and for the excellent remarks and improvement suggestions. Deborah Kaska is acknowledged for her fast and thorough help in language checking both with original publications and this dissertation.

My warmest appreciation goes to our entire group here in Oulu for creating a relaxed and helpful atmosphere, both current and former members. Thank you Ilkka Alahuhta, Otto Väyrynen, Ehsanul Hoque Apu, Carolina Bitu, Pirjo Åström, Pia Nyberg, Meeri Sutinen, Emma Pirilä, Krista Juurikka, Johanna Korvala, Maija Risteli, Tuukka Alanärä, Toni Sandvik, Carolina Bitu, Priscila Campioni, Tiago João, Wagner Gomes, Maurício Dourado and Marlene Vierthaler. I express my gratitude to the management and staff of Cancer and Translational Medicine Research Unit for enabling this work to be conducted in their facilities.

Naturally, all the other excellent co-authors are acknowledged for their valuable input in the original publications: Susanna Teppo, Marilena Vered, Heidi Holappa, Juha Parkkisenniemi, Tatiana Rinaldi, Petri Lehenkari, Reidar Grenman, Dan Dayan, Juha Risteli, Outi Renko, Sirpa Salo, Johanna Magga, Nilva K Cervigne, Raija Sormunen, Olli Vuolteenaho, Flávia Zandonadi, Adriana F. Paes Leme, Ricardo D Coletta, Heikki Ruskoaho, Joonas H Kauppila, Johanna Veijola, Rayan Mroueh, Saara Laitinen, Ylermi Soini, Veli-Matti Kosma, Iris-Sawazaki-Calone, Carolina Carneiro Soares Macedo and Risto Bloigu.

None of this work would have been possible without our skilled and helpful laboratory technicians: thank you Maija-Leena Lehtonen, Tanja Kuusisto, Eeva-Maija Kiljander, Maritta Harjapää, Sanna Juntunen, Merja Tyynismaa and Henna-Riikka Niskala.

Special thanks and appreciation goes to all the other doctoral students from DC Mikkola: Antti Tiisanoja (hopefully already a PhD when this is published), Maria Herrala, Otto Väyrynen and Matti Hannula for creating a pro-scientific atmosphere and means for peer-support. Keep up the good work!

I want to thank my parents and sister for supporting me in every turn along the way. Special acknowledgement goes to my father for providing an example and a goal for the schedule for thesis defense. Of course, I want to thank Lotta for enduring with me all these years it took to complete this study. I appreciate you for understanding and making compromises when needed.

This study was financially supported by Sigrid Juselius Foundation, Finnish Dental Society Apollonia, Paulo Foundation, K. Albin Johanssons Stiftelse, Ida Montin Foundation, Finnish Dental Foundation, Medical Research Center Oulu, The University of Oulu Scholarship Foundation and research funds from the Medical Faculty of the University of Oulu and Oulu University Hospital special state support for research.

Oulu, November 2017 Elias Sundquist

Abbreviations

α-SMA	alpha smooth muscle actin
APECED	autoimmune polyendocrinopathy – candidiasis – ectodermal dystrophy
ASR	age-standardized rate
Bax	Bcl-2 homologous antagonist X
Bcl-2	B-cell leukemia family protein 2
BD	budding-depth
CAF	carcinoma-associated fibroblast
CAIX	carbonic anhydrase 9
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
END	elective neck dissection
EPOR	erythropoietin receptor
FGF-2	fibroblast growth factor 2
FN	fibronectin
GLUT-1	glucose transporter 1
HGF	hepatocyte growth factor
HIF	hypoxia inducible factor
hMSC	human mesenchymal stromal cell
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
IIICTP	type III collagen C-terminal peptide
kDa	kilo Dalton
KM	Kaplan-Meier
MMP	matrix metalloproteinase
NOF	normal fibroblast
OR	odds ratio
OSCC	oral squamous cell carcinoma
OTSCC	oral tongue squamous cell carcinoma
PCNA	proliferating cell nuclear antigen
RM	rinsing media
SCC	squamous cell carcinoma
STAT-3	signal transducer and activator of transcription 3
TCA	trichloroacetic acid

TGF-β	transforming growth factor beta
TIF	tumor interstitial fluid
TME	tumor microenvironment
TNC	tenascin-C
TNM	tumor, node, metastasis
VEGF	vascular endothelial growth factor
WPOI	worst pattern of invasion

Original publications

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

I Teppo S, Sundquist E, Vered M, Holappa H, Parkkiseniemi J, Rinaldi T, Lehenkari P, Grenman R, Dayan D, Risteli J, Salo T* and Nyberg P* (2013) The hypoxic tumor microenvironment regulates invasion of aggressive oral carcinoma cells. Exp Cell Res 319(4): 376-389

II Sundquist E, Renko O, Salo S, Magga J, Cervigne N, Nyberg P, Risteli J, Sormunen R, Vuolteenaho O, Zandonadi F, Paes Leme A, Coletta R, Ruskoaho H and Salo T (2016) Neoplastic extracellular matrix environment promotes cancer invasion in vitro. Exp Cell Res 344(2): 229-240

III Sundquist E, Kauppila J, Veijola J, Mroueh R, Lehenkari P, Laitinen S, Risteli J, Soini Y, Kosma V-M, Sawazaki-Calone I, Macedo CCS, Bloigu R, Coletta RD and Salo T (2017) Tenascin-C and fibronectin expression divide early-stage tongue cancer into low- and high-risk groups. Br J Cancer 116(5): 640-648

* Equal contribution to the supervision of this study.

Contents

1 Introduction

Annually an estimated 300,400 new cases of oral cancers are diagnosed globally (data from 2012, including cancers of lip) and an estimated 145,000 oral cancer related deaths occur (Torre *et al*. 2015). Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer, and squamous cell carcinoma of the oral (or mobile) tongue (OTSCC) is the most prevalent OSCC. OTSCC is also the most aggressive of oral cancers and metastasizes at an early stage, yet is often diagnosed late (Bello *et al*. 2010a, Ganly *et al*. 2012). The overall survival of OTSCC remains at approximately 50% when OTSCCs diagnosed at any stage are accounted for (Marsh *et al*. 2011, Mroueh *et al.* 2017). However, even when OTSCC is diagnosed at an early stage when there are no clinical signs of metastases (cT1-T2N0), approximately 20% of those patients die of OTSCC (Ganly *et al*. 2012). This is proposed to occur due to the high invasion and metastasis properties of OTSCC and the occult nature of nodal metastases (Bello *et al.* 2010a, Kim & Cha 2012). The possible presence of occult metastases provides the rationale for elective neck dissection when nodal metastases are suspected. When elective neck dissection is performed on all cT1-T2N0 OTSCC patients the cancer specific survival is improved in comparison to watchful waiting and resecting the cervical lymph nodes only in cases of nodal relapse (D'Cruz *et al*. 2015). However, if elective neck dissection were performed on all the cT1-T2N0 OTSCC patients, then by subtraction 80% of them would be over treated and exposed to the substantial morbidities of neck dissection.

The tumor microenvironment (TME) has been recognized as a key player in cancer progression. TME consists of stromal cells, extracellular matrix (ECM) surrounding the cells and soluble factors embedded in the ECM and residing in the interstitial compartment. The properties of the TME influence the properties of cancer cells, and *vice versa*, creating a reciprocal dynamic interaction between these two entities and causing their co-evolution. Properties of the TME that effect cancer cells include oxygenation level, composition of interstitial fluid and ECM, and the types of residing host cells. The TME affects not only cancer cell invasion and metastasis but also the efficacy of treatment (Allen & Louise Jones 2011).

This study was designed to elucidate the effect of various TME factors on the behavior of cancer cells as well as their role in prognostication.

2 Review of the literature

2.1 Oral (mobile) tongue squamous cell carcinoma (OTSCC)

Oral cancer is the most common malignancy in the head and neck region, and 95% of oral cancers are squamous cell carcinomas (SCC) (Johnson *et al.* 2011). Oral, or mobile, tongue squamous cell carcinoma (OTSCC) is the most common cancer of the oral cavity covering approximately 25-40% of the intraoral malignancies (Bello *et al*. 2010a). The oral tongue includes the two anterior thirds of the tongue. The posterior third is called the base of the tongue, and cancers of this region are considered to be included in oropharyngeal cancers. In many studies the entity under examination is "oral cancer." This incorporates two distinct problems: first, there is variation between authors as to which anatomical areas are included in the oral cavity. Second, all the different cancers of the oral cavity are pooled into one group in order to increase the statistical power of the study. However, these different cancers, which are only linked by anatomical proximity, have different etiological and biological characteristics and thus should not be viewed as one type of cancer (Radoï & Luce 2013, Weatherspoon *et al.* 2015). This is the rationale behind focusing on squamous cell carcinoma of the oral tongue in this study. The epidemiological and etiological studies of oral cancers often fail to observe OTSCC as a distinct cancer. In the literature review of this thesis, information of OSCC is used when specific studies on OTSCC are lacking.

2.1.1 Incidence

In 2012 an estimated 300,400 new cases of oral cancers (including lip cancer) were diagnosed globally and an estimated 145,400 deaths were caused by oral cancer (Torre *et al.* 2015). These numbers vary substantially depending on the source and year (Franceschi *et al.* 2000, Torre *et al*. 2015, Warnakulasuriya 2009). OSCC is the eighth most common cancer worldwide, and incidence rates vary among men from 1 to 10 new cases/100,000 persons per annum (Petersen 2009). As is the case with most solid cancers, the incidence of OTSCC also increases with age. Additionally, incidence rates are higher among men when compared to women (Torre *et al.* 2015). However, a recent study reported an increase of OTSCC incidence among young white men and women in the United States using cancer incidence data from 1973 to 2012 (Tota *et al.* 2017).

The age standardized incidence of OTSCC in Finland has increased from 1.33 in 1955 – 1959 to 2.55 new cases/100,000 persons per annum. The increase is more notable among men: (Fig 1) from 0.81 in 1955 – 1959 to 1.59 new cases/100,000 persons in 2010 – 2014 (Finnish Cancer Registry). The incidence of OTSCC in Finland is among the lowest globally (Forman *et al.* 2013).

The incidence of OSCC, and OTSCC as well, is highly polarized geographically: oral cancer is more common in developing than in developed countries (Petersen 2009, Warnakulasuriya 2009). However, there are contradictory statistics showing a greater age-standardized rate (ASR) for the cancers of the oral cavity and lip among men and women in developed countries in comparison to developing countries (Torre *et al*. 2015). The highest incidence rates for OSCC are found in South and Southeast Asia, as well as parts of Latin America and the Caribbean, Pacific regions and parts of Western and Eastern Europe. For OTSCC the highest incidence rates are found in France, India and Brazil (Warnakulasuriya 2009). OSCC has been reported to be the number one cancer among men in India (Sankaranarayanan 1990), Sri Lanka (Ministry of Health. National Cancer Control Programme, Sri Lanka 2013), Pakistan and Bangladesh (Warnakulasuriya 2009). In Bhopal, India the ASR for OTSCC among men was 10.3/100,000 inhabitants per annum in 2004-2007, which is the highest incidence rate globally for that time period. The regions with the lowest ASR were found in Chile, Algeria and China, ASR being 0.2 in all of them (Forman *et al.* 2013).

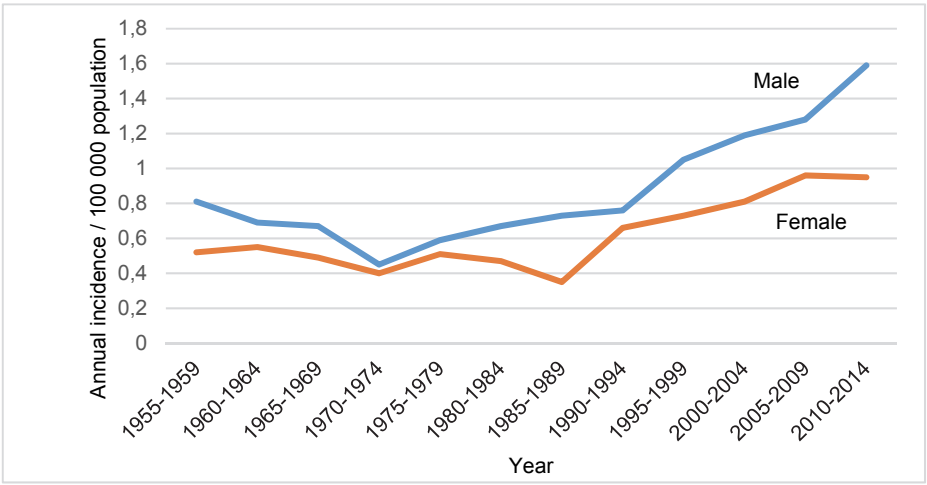

Fig. 1. Incidence of OTSCC in Finland from 1955 to 2014 (Finnish Cancer Registry).

2.1.2 Etiology

OTSCC is a multifactorial disease with a variety of known etiological factors (Table 1). The two most important etiological factors for OSCC as well as OTSCC are tobacco (in various forms) (Gandini *et al*. 2008) and alcohol (Baan *et al*. 2007, Lewin *et al*. 1998). Other risk factors include dietary intake, poor oral hygiene, trauma and viruses.

Alcohol consumption is shown to increase the risk of developing OSCC in a dose-dependent manner (Ng *et al.* 1993, Vokes *et al.* 1993), which is also the case with other cancers (Room *et al*. 2005). Ethanol is oxidized by alcohol dehydrogenase to acetaldehyde that is the major mediator of the carcinogenic effect of alcohol (Baan *et al.* 2007). The carcinogenic effect of alcohol consumption on different subsites of oral cavity is sparsely studied, but it has been found that alcohol consumption has significantly greater impact on developing SCC on the floor of the mouth and retromolar trigone when compared to the tongue (Boffetta *et al*. 1992, Jovanovic *et al.* 1993, Llewelyn & Mitchell 1994). However, there are studies with controversial results with similar odds ratios (ORs) for tongue and other oral cavity subsites (Franceschi *et al*. 1992, Franco *et al.* 1989, Hayes *et al*. 1999, Znaori *et al*. 2003). In addition to consuming alcohol by ingesting, also long lasting continuous use of mouthwashes with over 25 vol% alcohol content is shown to increase the risk of developing OSCC (McCullough & Farah 2008, Winn *et al.* 2001), although there are contradictory results (Boyle *et al.* 2014).

Cigarette smoking remains the most common form of tobacco consumption. Alongside alcohol consumption, smoking is one of the two main etiological factors attributing to most of the OSCC cases. Additionally, the dose-dependent relationship between smoking and incidence of OSCC is well documented (Hashibe *et al.* 2007, Lewin *et al.* 1998). Other forms of consuming tobacco with combustion include cigar and pipe smoking, which cause OSCC as well (Franceschi *et al.* 1990, Garrote *et al*. 2001). The risk of developing OSCC increases alongside smoking frequency, duration of consumption and cumulative lifetime consumption regardless of the form of tobacco smoking (cigarette, cigar or pipe) (Radoï & Luce 2013). However, in the case of OTSCC, smoking exclusively dark tobacco has been found to be more harmful than smoking exclusively blond tobacco (Oreggia *et al.* 1991).

As is the case with tobacco smoking and alcohol drinking separately, additionally the joint effect of these factors is widely studied. In concomitant use, these two risk factors have a synergistic dose-dependent effect, the most affected

site in the oral cavity being the floor of the mouth (Lubin *et al*. 2009, Radoï *et al.* 2013a).

The category "smokeless tobacco" contains a vast variety of different products and hence it cannot be discussed as one product entity. The most common products of this category used in Western countries are chewing tobacco, as well as moist and dry snuff. These are primarily used in the USA and Scandinavia and are somewhat homogenous groups (Rodu & Jansson 2004). On the other hand, smokeless tobacco used for example in India is often produced by small companies or even individual farmers, leading to compromised control of fermentation and curing, and more carcinogenic molecules are produced as a result (Brunnemann *et al*. 1985, Rodu & Jansson 2004). Controversy remains as to whether smokeless tobacco used in the USA and Scandinavia – snuff – increases the risk of oral cancer. The consensus of Finnish Current Care Guideline declares that snuff might increase the risk of OSCC (Oral cancer: Current Care Guideline 2012). There are studies showing that oral snuff does not increase the risk for OSCC (Lewin *et al*. 1998, Luo *et al*. 2007, Rosenquist *et al.* 2005b, Schildt *et al*. 1998b), and on the other hand, some studies have shown that it does (Roosaar *et al*. 2008, Winn *et al.* 1981) with an incidence rate ratio of 3.1 (95% confidence interval 1.5 – 6.6) for the combined incidence of oral and pharyngeal cancers (Roosaar *et al.* 2008). However, when interpreting the results from these studies, especially older ones, it has to be considered that the amount of carcinogens at least in Swedish snuff has been shown to be decreased from the 1980s to the early 2000s (Österdahl *et al.* 2004). Additionally, it has been speculated that since Swedish moist snuff is a non-fermented product that undergoes heat treatment, it is free of microorganisms, which lowers the risk for formation of nitrate and nitrosamines (Radoï & Luce 2013). Other forms of smokeless tobacco include betel quid that is prepared from areca nuts. Betel quids can be prepared with or without tobacco. Betel quid consumption, as well as areca nut consumption alone, is shown to increase the incidence of OSCC and this carcinogenic effect is independent of the betel quid tobacco content (Nair *et al*. 2004, Thomas *et al.* 2007).

Dietary intake is found to be associated with increased risk for OSCC and it has been considered to be an underlying effector in about 30% of cancers in Western countries (Key *et al.* 2004). One of the most important dietary related aspects linked to increased OSCC incidence is reduced fruit and vegetable intake (Pavia *et al.* 2006), along with increased consumption of red meat (Levi *et al*. 1998). The protective role of vegetable and fruit intake is indisputable, and moreover, it has been suggested to modulate the carcinogenic effects of tobacco and alcohol

(Kreimer *et al.* 2006). In addition to vegetable and fruit consumption, coffee and tea consumption have been shown to decrease the incidence of OSCC. Similarly to vegetables and fruits, the protective mechanism for coffee and tea is suggested to be an antioxidant effect that repairs cellular damage. In the case of especially green tea the mechanism has been suggested to be related to polyphenols that induce apoptosis of carcinoma cells as well as inhibit their growth and invasion. Coffee or tea consumption alone is associated to reduced risk for OSCC, and moreover together their protective effect is multiplicative (Beltz *et al*. 2006, Hsu *et al.* 2002, Li *et al.* 2016, Radoï *et al*. 2013c, Zhang *et al*. 2015).

Interestingly, existing evidence suggests that increased body mass index protects from developing OSCC. However, the underlying mechanisms are yet to be elucidated (Kreimer *et al.* 2006, Nieto *et al*. 2003, Radoï & Luce 2013).

Poor oral hygiene and dental status have been identified as independent risk factors for OSCC in various studies (Balaram *et al*. 2002, Bundgaard *et al.* 1995, Garrote *et al.* 2001, Lissowska *et al.* 2003, Rosenquist *et al*. 2005a, Subapriya *et al*. 2007, Zheng *et al*. 1990). In contrast, some studies have found oral hygiene not to impact the risk of developing OSCC, or to have only a minimal impact (Lewin *et al*. 1998, Marshall *et al.* 1992, Talamini *et al.* 2000). However, in some studies that did not find a connection, oral hygiene was only assessed with a questionnaire using *e.g.* number of toothbrushes used and number of visits to the dentist annually as indicators, while studies associating poor oral hygiene to OSCC included clinical examination of oral hygiene in their methodology. In addition to the status of oral hygiene and dentition, also infections of the oral cavity have been linked to OSCC (Schildt *et al*. 1998a).

Trauma or local irritation from *e.g.* dentures of fillings that cause sores are considered a risk factor for OTSCC alongside poor oral hygiene (Singhvi *et al.* 2017, Velly *et al.* 1998).

The role of human papilloma virus (HPV) infection in OTSCC has been studied, and evidence suggests that it may not play a role in tongue cancer (Kantola *et al*. 2000, Liang *et al.* 2008, Mirghani *et al.* 2015, Poling *et al*. 2014, Sgaramella *et al.* 2015), although there is also evidence to the contrary (Miller & Johnstone 2001, Mork *et al.* 2001, Syrjänen *et al.* 2011). The role of HPV in carcinogenesis is more obvious in cancers originating from the base of the tongue (Dahlgren *et al.* 2004, Hansson *et al.* 2005, Jiang *et al.* 2015, Nordfors *et al.* 2014). In addition to HPV infection, oral candidiasis and herpes infection have been studied for their association to increased risk of OSCC but the results are highly contradictory

(Garrote *et al.* 2001, Macfarlane *et al.* 2012, Radoï *et al.* 2013b, Rosenquist *et al*. 2005a, Schildt *et al.* 1998a, Talamini *et al.* 2000).

Family history of head and neck squamous cell carcinoma (HNSCC) has been shown to increase the risk for developing OSCC (Brown *et al*. 2001, Foulkes *et al*. 1995, Garavello *et al.* 2008, Radoï *et al.* 2013b).

In addition to family history, also other genetic factors are related to increased risk for OSCC. These include alterations in genomic copy numbers and other genetic alterations *e.g.* leading to the presence of potentially malignant disorders (Ali *et al.* 2017, Bhattacharya *et al*. 2011, Salahshourifar *et al.* 2014). Some diseases and syndromes increase the risk for OSCC, including Fanconi anemia, dyskeratosis congenital and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome (Rautemaa *et al*. 2007, Vigneswaran & Williams 2014).

Some cases of OTSCC cannot be explained by known etiological factors, but are rather an unfortunate outcome of random mutations (Kumar *et al.* 2016).

Table 1. Etiological factors of OTSCC.

Factor	Est. strength of evidence	Reference
Alcohol consumption	Good	Baan et al. 2007
Mouthwashes w/ > 25 vol% alcohol content	Moderate	McCullough & Farah 2008
Tobacco smoking	Good	Gandini et al. 2008
Snuff	Poor	Roosaar et al. 2008
Betel quid/areca nut	Moderate	Thomas et al. 2007
Other smokeless tobacco	Poor	Rodu & Jansson 2004
Poor dietary intake	Moderate	Pavia et al. 2006
Poor oral hygiene	Moderate	Subapriya et al. 2007
Trauma or local irritation	Moderate	Singhvi et al. 2017
Human papilloma virus	Poor	Syrjänen et al. 2011
Family history/genetic factors	Moderate	Radoï et al. 2013b, Vigneswaran & Williams 2014

2.1.3 Diagnosis

Early diagnosis and treatment of OTSCC are of utmost importance in order to avoid substantial morbidities through large resections and to achieve low mortality.

Delays in diagnosis and treatment can be divided into primary, secondary and tertiary delays. Primary delay is the delay from onset of symptoms to seeking medical attention and is thus patient dependent. Secondary delay is the delay of primary healthcare provider referral of the patient to a hospital that treats OTSCC, and is thus healthcare provider dependent. Finally, the tertiary delay is the delay of the treating hospital from referral to treatment (Joshi *et al.* 2014).

OTSCC has a tendency to be asymptomatic at early stages, which leads to increased primary delay (Guggenheimer *et al.* 1989). The increased delay in turn leads to the fact that as many as 42 – 64% of all OTSCC cases are diagnosed at advanced stages (stages III – IV, Tables 2 and 3) (Chen *et al.* 2008, Goldstein *et al.* 2013, Ling *et al.* 2013, Mroueh *et al.* 2017, Thiagarajan *et al.* 2014).

OTSCC has conventionally been diagnosed with biopsy that has been acquired based on clinical examination. Other diagnostic tools have been developed as well [reviewed in Epstein *et al*. (2002) and Omar (2015)]. These include brush biopsy (Gupta *et al.* 2007), vital tissue staining (Gupta *et al.* 2007), genetic biomarkers (Rosin *et al.* 2000), light-based detection systems (Guze *et al.* 2015, Swinson *et al.* 2006), DNA content analysis (Kaur *et al*. 2016, Ma *et al.* 2014) and salivary biomarkers (Gualtero & Suarez Castillo 2016, Hu *et al.* 2008). These other methods are for the most part under development and/or are used as adjuvant methods alongside biopsy, *e.g*. toluidine blue staining to detect the most dysplastic area for biopsy. While biopsy remains a gold standard in OTSCC diagnosis, it has its clear limitations: biopsy requires trained personnel, is expensive and relatively time consuming and owing to its invasive nature is not suitable for screenings (Brinkmann *et al*. 2011).

The most used classification among all solid cancers is the tumor, node, metastasis (TNM)-classification, and this differs for cancers located in different anatomical sites. The TNM-classification assigned based on clinical evaluation is referred to as clinical TNM (cTNM); after surgery, based on pathological findings, it is referred to as pathological TNM (pTNM). Tumor (T) is based on the size and anatomical involvement of the primary tumor; node (N) is based on the presence, number, size and location of regional lymph node metastases, and metastasis (M) is based on the presence or absence of distant metastases. The TNM-classification for head and neck cancers is specified in Table 2. Cancers can be divided into stages from I to IV based on the TNM classification, clinical or pathological (Table 3). In this study, cTNM is used and stages I and II are considered as early-stages.

2.1.4 Treatment modalities

Despite all the advances in surgery, radiotherapy and chemotherapy, the 5-year survival of OSCC still remains at approximately 50%, 80% in early-stage (T1 – T2N0; stage I – II) cases and 20% in late-stage cases (Marsh *et al*. 2011, Mroueh *et al.* 2017, van der Waal *et al.* 2011).

As in treating any disease, the two most important guidelines in treating OTSCC are to provide sufficient treatment, and on the other hand avoid overtreatment. Treatment should be tailored individually to each patient aiming for maximal preservation of function and quality of life as well as survival of the patient. Patients with early-stage OTSCC often receive a single-modality treatment: surgical resection of the tumor or radiotherapy alone. Of these two modalities, surgical resection is preferred and remains the cornerstone of OTSCC treatment (Werning cop. 2007). Radiotherapy as a single modality treatment or with chemotherapy can be applied in cases of large and/or inoperable tumors.

In early-stage OTSCCs surgical treatment is often sufficient. However, there is a subgroup of patients among those with early-stage OTSCC whose cancer is more aggressive, yet the contemporary diagnostic tools are incapable of detecting those individuals — hence the 20% mortality of early-stage OTSCC patients. From early-stage patients, sentinel lymph node biopsies can be taken in selected cases (Paleri *et al*. 2005, Oral Cancer: Current Care Guideline 2012). Also elective neck dissection (END) can be performed if the primary tumor is large or occult lymph node metastases are expected to be present, *e.g*. in cases with deep depth of invasion or thick tumor (O'Brien *et al.* 2003, O-charoenrat *et al.* 2003). Indeed, some advocate END because the incidence of occult lymph node metastases is high even in early-stage OTSCC; the incidence of regional recurrence in N0 neck has been reported to be 10 – 47% (Huang *et al.* 2008, Wensing *et al.* 2006, Yuen *et al.* 1997, Yuen *et al*. 1999). Survival is improved among those patients that undergo END in contrast to those who undergo therapeutic neck dissection as a salvage operation (Abu-Ghanem *et al*. 2016, D'Cruz *et al.* 2015). However, naturally surgery-related morbidities are increased among these patients, and there is no clear consensus on the benefit of END *vs*. watchful waiting.

Radiotherapy can be applied as adjuvant therapy in cases of large primary tumors, positive or close surgical margins, and signs of perineural, lymph and/or vascular invasion. Radiotherapy irritates tissue making it more fibrotic and slow healing. Thus radiotherapy is typically applied postoperatively (Omura 2014).

Chemotherapy can be added in advanced cases, but alone it is not curative. Combined treatment with radiotherapy and chemotherapy – chemo radiotherapy – as adjuvant treatment before or after surgery has increased the survival rate of patients with non-metastatic OSCC marginally (Omura 2014). However, there are increased treatment related morbidities associated to this treatment modality (Klein *et al.* 2014).

Table 2. TNM-classification of oral cancers (TNM Classification of Malignant Tumors, 7th Edition).

TNM	Criteria
T	Primary tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ (non-invasive carcinoma)
T1	Tumor ≤ 2 cm
T2	Tumor > 2 cm but ≤ 4 cm
T3	Tumor > 4 cm
T4a	Tumor invades through cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus or skin of face
T4b	Tumor invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery
N	Regional lymph nodes (cervical 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
N2a	Metastasis in a single ipsilateral lymph node, > 3 cm but ≤ 6 cm
N2b	Metastasis in multiple ipsilateral lymph nodes, ≤ 6 cm
N2c	Metastasis in bilateral or contralateral lymph nodes, ≤ 6 cm
N3	Metastasis in lymph node > 6 cm
M	Distant metastasis
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 3. Staging of oral cancers.

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

2.2 Prognostic factors in OTSCC

Prognostic factors can be divided into demographic, clinical, histopathologic and immunohistochemical factors. Precise diagnosis and prognostication at early-stage OTSCC would be highly advantageous for two major reasons: for utilization of the most effective treatment modalities and multimodality treatment for those at high risk of adverse outcome, and on the other hand sparing those with better prognosis from overtreatment and adverse effects of aggressive treatments.

2.2.1 Demographic and clinical prognostic factors

Demographic prognostic factors include variables such as patient age (Davidson *et al.* 2001), gender (Berrino & Gatta 1998, Dickman *et al.* 1999), socioeconomic status (Hagedoorn *et al.* 2016, Lee *et al*. 2012) and obesity (Iyengar *et al.* 2014, Iyengar *et al.* 2016), as well as those related to lifestyle including tobacco, betel and alcohol consumption.

Clinical prognostic factors include TNM-classification (Edge & Compton 2010), tumor depth (Jung *et al.* 2009, Pentenero *et al.* 2005, Thiagarajan *et al.* 2014), appearance and growth type of the tumor (Bonnardot *et al.* 2011, Sharma *et al.* 2013), midline involvement (Kurita *et al.* 2004, Lloyd *et al.* 2012), recurrence (Peng *et al.* 2014, Yuen *et al.* 1997), second primary (van der Waal & de Bree 2010), metastasis (Ganly *et al.* 2012, Goodman *et al.* 2009) and comorbidities (Paleri *et*

al. 2010). The most important prognostic factor in OTSCC (and other head and neck carcinomas) is the presence of lymph node metastasis. The presence of lymph node metastasis decreases the survival rate by *ca.* 50% and increases the odds of distant metastasis (Leemans *et al*. 1993, Leemans *et al.* 1994, O'Brien *et al.* 2003).

2.2.2 Histopathological prognostic factors

Histopathological prognostic factors of OSCC (Table 4) include tumor grading, malignancy grading score, worst pattern of invasion (WPOI), perineural invasion, lymphovascular invasion, muscle invasion, surgical margin status (malignancy or dysplasia at margin), and tumor budding and depth (BD-model) (Almangush *et al*. 2014, Anneroth *et al*. 1987, Bello *et al*. 2010a, Bello *et al.* 2010b, Brandwein-Gensler *et al*. 2005, Broders 1920, Loree & Strong 1990, Myers *et al*. 2000, Soo *et al*. 1986, Sparano *et al.* 2004, Weijers *et al.* 2002, Weijers *et al.* 2009). As these prognosticators work on the general case of OSCC, they fail to predict the outcome of more precise patient groups and more importantly; excluding WPOI, tumor budding and depth of invasion, are not capable of differentiating patients with early-stage OTSCC into those with more aggressive disease who are at a great risk of adverse outcome and those prone to a more favorable outcome (Almangush *et al.* 2014).

Table 4. Histopathological prognostic factors in OSCC.

Prognostic factor	Reference
Tumor grading	Broders 1920
Malignancy grading	Anneroth *et al.* 1987
Worst pattern of invasion	Brandwein-Gensler *et al.* 2005
Perineural invasion	Soo *et al.* 1986
Lymphovascular invasion	Myers *et al*. 2000
Muscle invasion	Sparano *et al.* 2004
Margin status	Loree & Strong 1990, Weijers *et al.* 2002
Budding and depth	Almangush *et al.* 2015

Tumor grading

Tumor grading is a histopathological system of dividing squamous cell carcinomas into three categories based on the degree of cancer cell differentiation. This system

accounts for the degree of keratinization, cellular and nuclear pleomorphism and mitotic activity. Tumors are divided into low, intermediate or high grade with low grade being the most and high grade least differentiated. This grading system was originally introduced by Broders (1920) and it is based on the assumption that the least differentiated cells are the most aggressive and thus adversely affect survival. The predictive value of this grading system in OSCC is somewhat controversial.

Malignancy grading

The first malignancy grading system was introduced by Jakobsson *et al*. (1973). Their system accounted for tumor structure, mode and stage of invasion, degree of keratinization, mitoses, nuclear pleomorphism, vascular invasion and cellular response. In conclusion, this system is only based on the properties of the tumor itself.

After this, Anneroth *et al.* (1987) introduced a new system for OSCC malignancy grading. Their system is based on both tumor cells and more importantly on tumor-host interaction. Their system accounts for degree of keratinization, nuclear pleomorphism, number of mitoses, pattern and depth of invasion, and inflammatory cell infiltration.

Moreover, Bryne *et al.* (1989) continued to add specificity of malignancy grading by introducing a model that is based on the cancer cells in the invasive front. Their model is based on the nuclear pleomorphism, degree of keratinization, number of mitoses, pattern of invasion and inflammatory cell infiltration at the invasive front.

Histologic risk assessment score

The histologic risk assessment score as proposed by Brandwein-Gensler *et al*. (2005) includes three variables: perineural invasion, lymphocytic infiltrate at the tumor-host interface and worst pattern of invasion at the tumor-host interface. Based on these variables the patients can be divided into low-, intermediate- and high-risk groups. This score has been shown to associate with local recurrence in OTSCC (Vered *et al*. 2010).

Muscle invasion

Along with perineural and lymphatic invasion, also muscle invasion has been found to be a predictor of recurrence in OTSCC (Sharma *et al.* 2013). Additionally, in their study of multiple predictors for early-stage OTSCC, Sparano *et al*. (2004) found, among other variables, the depth of muscle invasion to be a predictor for occult neck metastasis.

Margin status

The involvement of cancer cells in or at the proximity of surgical margins is widely analyzed from glossectomy samples, and involved or close margins are used as indicators for adjuvant therapy (Montero *et al*. 2014). To support this, the margin status has been found to be a predictor of local control in early-stage OTSCC (Chang *et al*. 2013). However, there is controversy regarding whether close margins should be considered an indication for adjuvant therapy. Ch'Ng *et al.* reported that surgery alone provided acceptable local control of OSCC if close but uninvolved margins were the only indicator of adverse outcome (Ch'Ng *et al*. 2013).

Besides cancer cell involvement, also the presence of dysplasia at surgical margins has been studied and found to be a prognosticator of adverse outcome in OTSCC (Sharma *et al.* 2013).

Budding and depth

Tumor cell budding (presence of cancer cell clusters of less than five cells) and depth of invasion have been studied earlier and found to be independent prognosticators in OTSCC (Ganly *et al.* 2012, Wang *et al*. 2011). More recently, Almangush *et al.* (2015) introduced a model for prognostication of early-stage OTSCC patients based on both tumor budding and depth: the BD-model.

2.2.3 Immunohistological prognostic factors

Immunohistological factors have been studied rigorously and new prognosticators seem to emerge at regular intervals. However, it is difficult to distinguish the ones that have the most potential to become routine tools of pathology as studies conducted to confirm the results of novel prognosticators are few or lacking (Rivera *et al.* 2017). Immunohistochemical prognostic factors studied in OSCC include

alpha-smooth muscle actin [α-SMA, indicates cancer-associated fibroblasts (CAFs)] (Bagordakis *et al.* 2016, Bello *et al.* 2011, Kellermann *et al.* 2007), proteins related to self-sustaining growth [cyclin D1 and signal transducer and activator of transcription 3 (STAT-3)] (Noorlag *et al.* 2015, Xie *et al*. 2015), tumor angiogenesis markers [vascular endothelial growth factor (VEGF)] (Chuang *et al*. 2006), proliferation markers [Ki-67, proliferating cell nuclear antigen (PCNA)] (Kato *et al.* 2011, Wangsa *et al.* 2008), apoptosis markers [B-cell leukemia family protein 2 (Bcl-2) and Bcl-2 homologous antagonist X (Bax)] (De Vicente *et al.* 2006), epidermal growth factor receptor (EGFR) family (Ryott *et al.* 2009), matrix metalloproteinases (MMPs) (Kawano & Yanagisawa 2006, Kim *et al*. 2006, Korpi *et al*. 2008), tumor suppression markers (p53, p14ARF, p16INK4A, p21, p27, pRb, maspin) (Goto *et al.* 2005, Isayeva *et al*. 2015, Kato *et al*. 2011, Kwong *et al*. 2005, Mineta *et al.* 1999, Po Wing Yuen *et al.* 2001, Soni *et al.* 2005, Verma *et al*. 2014, Yasumatsu *et al.* 2001), adhesion markers (E- and N-cadherin, CD44, versican, β-catenin) (da Silva *et al.* 2015, Lee *et al.* 2015, Li *et al.* 2009, Pukkila *et al.* 2007, Yao *et al*. 2017), hypoxia markers [hypoxia-inducible factor (HIF) -1 and -2, carbonic anhydrase-9 (CAIX), glucose transporter 1 (GLUT-1), erythropoietin receptor (EPOR)] (Roh *et al.* 2009, Woolgar 2006) and inflammatory response (Sanoglu *et al.* 1994). Of these markers, the most promising seem to be cyclin D1, STAT-3, p53, p21, p27, cadherins and MMPs (Sinevici & O'sullivan 2016).

2.3 Hypoxia

Both rapidly proliferating tumor cells and tumor infiltrating host cells increase the oxygen consumption in a cancer tumor as well as increase its size and thus diffusion distances (Siemann 2011). However, the rapidly developing neovasculature is often malformed and leaky, and together with increased diffusion distances this leads to distortion of the balance between oxygen demand and supply, thus forming areas of hypoxia in the tumor (Carmeliet 2005). Hypoxia is known to be a significant contributor to initiation and maintenance of aggressive cell behavior by changing gene transcription and thus affecting cell metabolism, cell cycle, invasion and angiogenesis (Harris 2002). In addition to cell behavior, hypoxia affects the composition of the ECM through its effects on stromal cells leading to *e.g.* accumulation of ECM components – such as tenascin-C (TNC) – via suppression of MMP activity (Gebb & Jones 2003, Orend & Chiquet-Ehrismann 2006).

CAIX is a transmembrane enzyme that functions in the regulation of pH homeostasis via reversible hydration of CO_2 (Lou *et al.* 2011). In addition, CAIX

has a role in cell proliferation and transformation, adhesion and tumorigenesis. CAIX is found in various solid tumors, including head and neck cancers. The expression of CAIX in cancer is restricted to hypoxic and perinecrotic areas (Janssen *et al.* 2005, Wykoff *et al.* 2000). CAIX is suggested to have a role in adaptation to hypoxia and the presence of CAIX in some tumors is correlated to poor prognosis (Lou *et al.* 2011, Potter & Harris 2003, Swinson *et al.* 2003).

Hypoxia has been simulated using various different methods, *e.g.* hypoxia chamber or cobalt chloride ($CoCl_2$). $CoCl_2$ mimics hypoxia via disruption of HIF-1 and -2 pathways, which are the main mediators of the effect of hypoxia at the cellular level. $CoCl_2$ is comparatively easy to use and thus has gained popularity in hypoxia assays (Yuan *et al.* 2003). Another method to create a hypoxic environment is to use a hypoxia chamber. While a hypoxia chamber naturally provides a precise method of influencing the pO_2, it creates an unnatural distribution of oxygen supply in tissue: *in vivo* there is a gradient of oxygen available in tissue with a decreasing amount when approaching the center of non-vascularized area. When using a hypoxia chamber, the hypoxic atmosphere creates a homogenous hypoxia in the tissue discs. A more natural gradient of oxygen can be created using microfluidic systems. Although they mimic the oxygen gradient of tissue microenvironment more accurately, they require bulky control instruments and numerous interconnections thus limiting the practical applicability (Brennan *et al.* 2014).

2.3.1 Role of hypoxia in cancer progression

Even though hypoxia limits the growth of tumors, it additionally provides a stimulus for cancer invasion and metastasis and thus acts as an independent predictor of poor prognosis. Indeed, tumor oxygenation has been shown to adversely affect the survival of cancer patients in various cancers, including cancers of head and neck at advanced stages (Brizel *et al*. 1997, Nordsmark & Overgaard 2000).

Cellular response to hypoxia is mediated mainly through HIF-1, which consists of two sub-units: HIF-1α and HIF-1β. HIF-1β is stable, but HIF-1α is degraded under normoxia, and this degradation is oxygen dependent. In hypoxia the two subunits can form the HIF-1-complex which functions in the nucleus by binding to hypoxia responsive elements in DNA of the target genes and acts as an activating transcription factor for more than 100 genes that induce key aspects of tumorigenesis. Most of these genes are involved in adaptation to hypoxia and regulate processes such as formation of growth factors, glycolytic enzymes,

angiogenesis factors, vasoactive peptides, as well as genes involved in proliferation, apoptosis and invasion (Rankin & Giaccia 2016). Additionally, hypoxic exposure leads to stiffening of the ECM through increased cross-linking of ECM proteins. The stiffening of the ECM increases cancer cell invasion and angiogenesis (Lu *et al.* 2012).

Hypoxic exposure can be divided into chronic and short-term or fluctuating depending on the time of exposure. The exposure is considered chronic when it lasts for over 24h. Chronic and fluctuating hypoxic exposures have been shown to affect cellular functions differently. Additionally, specifically fluctuating hypoxia has been suggested to be needed for the induction of invasion and in stem cell maintenance (Dai *et al*. 2011, Dewhirst 1998, Postovit *et al.* 2008, Subarsky & Hill 2008, Sun *et al.* 2011). However, there are also studies suggesting that chronic hypoxia is the main promoter of a more aggressive phenotype (Alqawi *et al.* 2007). This shows that the effect of hypoxia might be dependent on the cell type and their microenvironment.

Since hypoxia is closely related to all solid cancers and modifies the behavior of cancer cells, it has naturally been studied as a prognostic factor. Indeed, tumor hypoxia has been found to be a prognosticator in OSCC with high hypoxic levels indicating poor prognosis. These results were obtained by using HIF-1α as a hypoxia marker (Eckert *et al.* 2010, Pérez-Sayáns *et al.* 2011). In addition to causing poor prognosis by altering the cancer cell phenotype towards more aggressive behavior, hypoxia causes tumors to become resistant to chemo- and radiotherapy (Janssen *et al*. 2005).

2.4 Tumor microenvironment (TME)

The stroma surrounding a cancer tumor is called the tumor microenvironment (TME). The role of the TME for cancer development has become abundantly clear. The cells and ECM components are crucial for the proliferation and invasion of cancer cells through receptor mediated, dynamic interactions between cancer cells and ECM molecules (Metwaly *et al*. 2012). Together these components create a complex and dynamic environment that is in constant reciprocal crosstalk with cancer cells and changes alongside the tumor progression. The TME in different cancers varies in its composition as well as in its effect on prognosis, and cancer cell migration and invasion (Mueller & Fusenig 2004).

2.4.1 Composition

The TME consists of stromal cells, including CAFs, endothelial cells and immune cells, ECM, including proteins such as collagens, elastin, tenascin-C and fibronectin (FN), and other extracellular molecules that can be embedded in the ECM or exist in a soluble form in interstitial fluid including growth factors, proteases, protease inhibitors and other signaling molecules, as well as extracellular vesicles and other soluble factors (Koontongkaew 2013, Fig 2).

The TME has been mimicked utilizing various methods reviewed by Wu & Melody (2014). Probably the most popular method to study cancer cell invasion in the TME model is to use Matrigel® embedded with human fibroblasts (Emonard *et al.* 1987). Similarly, collagen gels have been used as well as various spheroid and sandwich assays (Åkerfelt *et al*. 2015, Ghosh *et al*. 2007, Korff & Augustin 1999). The common problem with these methods is that they utilize tissue from different species; Matrigel® is manufactured using mouse tumor and collagens used in these assays originate most commonly from rat tail. Thus they do not mimic properly the human TME.

2.4.2 Role of TME in cancer progression

Originally the ECM component of the TME was considered a stable, space-filling scaffold and thus a barrier against cancer invasion. The ECM is now recognized to have an important reciprocal role in cancer progression and it is actively altered during carcinogenesis. At early stages of cancer progression the genetic alterations in cancer cells activate the mechanisms that lead to secretion of growth factors, cytokines and ECM proteins that start the transformation of ECM as well as the rest of the TME into a more pro-tumorigenic milieu. This is accomplished by increasing the protective role of the TME from host immunity as well as increasing the pro-tumorigenic signaling from the adjacent, newly transformed stromal host cells. Taken together the TME affects most of the hallmarks of cancer (Hanahan & Weinberg 2011).

Since the dynamic role of the ECM has been established, various components of ECM have been studied to identify the key factors in the biological processes underlying the carcinogenic transformation, cancer invasion and metastasis, as well as prognostication and targets for therapeutic intervention. These proteins include, among others, TNC and FN (Allen & Jones 2011).

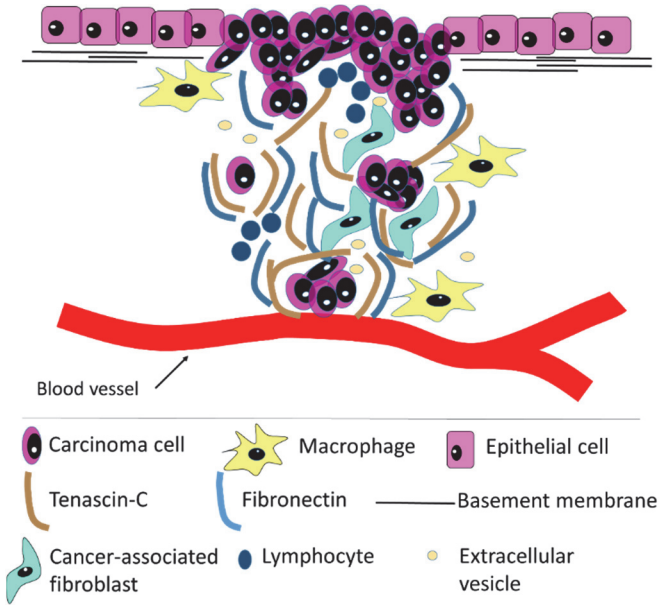

Fig. 2. Composition of TME. TME consists of stromal cells (*e.g.* macrophages lymphocytes and cancer associated fibroblasts), ECM (containing *e.g.* tenascin-C and fibronectin) and soluble factors (*e.g.* extracellular vesicles).

2.5 Tenascin-C (TNC)

2.5.1 Tenascin-C in cancers

TNC (Fig 3) is an ECM glycoprotein that binds to a variety of other ECM molecules including fibronectin, periostin, integrins and collagens (Venning *et al*. 2015). In physiological conditions TNC is found primarily in developing embryonic tissues, especially in those related to the epithelial – mesenchymal transition (EMT) and to cell migration pathways. In adult tissues, TNC is expressed exclusively during inflammation, wound healing and cancer progression (Juhász *et al.* 2000, Orend & Chiquet-Ehrismann 2006). TNC is upregulated in both transformed epithelial and stromal cells (Emoto *et al.* 2001, Orend & Chiquet-Ehrismann 2006). Increased stromal TNC expression is correlated with poor prognosis or aggressive phenotype in several cancers, including breast (Ishihara *et al.* 1995, Jahkola *et al*. 1998), colorectal (Emoto *et al.* 2001, Sis *et al.* 2004), esophageal (Yang *et al.* 2016), bladder (Brunner *et al*. 2004) and Merkel cell carcinomas (Koljonen *et al.* 2005);

glioma (Herold-Mende *et al.* 2002), malignant pleural mesothelioma (Kaarteenaho-Wiik *et al.* 2003), glioblastoma multiforme (Leins *et al.* 2003), malignant melanoma (Kääriäinen *et al.* 2006), and astrocytoma (Varga *et al.* 2012). Additionally, radiolabeled TNC antibody treatment has been successfully studied and found to increase survival of patients in glioblastoma multiforme and anaplastic astrocytoma in a phase II clinical trial (Reulen *et al.* 2015). A slight increase in survival has also been obtained in a phase II clinical trial of malignant glioma (Reardon *et al*. 2002).

2.5.2 Tenascin-C in oral squamous cell carcinoma

The presence of TNC in OSCC cells and stroma has been studied, and TNC has been found in the stroma of potentially malignant oral lesions as well. Additionally, the amount of TNC in the stroma of those lesions has been found to increase alongside dysplasia (Tiitta *et al*. 1994). In OSCC TNC is produced by either the OSCC cells themselves (Driemel *et al.* 2007) or by the surrounding host mesenchymal fibroblast-like cells (Hindermann *et al.* 1999, Metwaly *et al.* 2012).

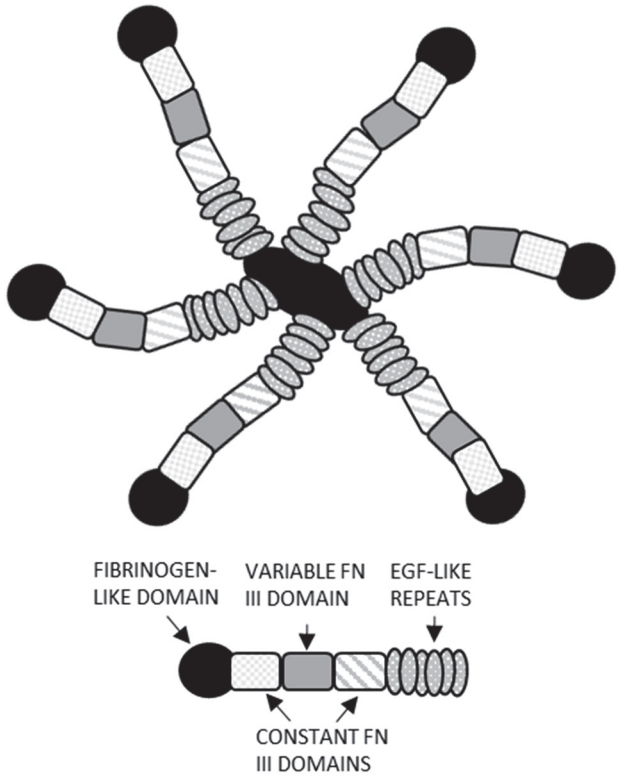

Fig. 3. Schematic illustration of the structure of TNC.

2.6 Fibronectin (FN)

2.6.1 Fibronectin in cancers

FN (Fig 4) is a large (~440 kDa) heterodimeric glycoprotein that is essential in development, wound healing and normal cell function. FN exists in soluble and insoluble forms. The soluble form is found in blood plasma and the insoluble form is an essential part of the ECM. FN is produced by a variety of cells, but mainly fibroblasts and it is organized as a fibrillary network. FN is involved in the

regulation of a variety of cellular activities including cell adhesion, migration, growth and differentiation. The effect of FN is mediated through interactions with cell surface integrin receptors (Mao & Schwarzbauer 2005, White *et al.* 2008).
FN is shown to be involved in carcinogenesis, including cancer invasion and metastasis (Stivarou & Patsavoudi 2015). Stromal FN has been found to correlate with poor prognosis or aggressive phenotype of the cancer *e.g.* in urothelial carcinoma (Ioachim *et al.* 2005), breast cancer (Ioachim *et al.* 2002) and colorectal cancer (O'Shannessy *et al.* 2014).

2.6.2 Fibronectin in oral squamous cell carcinoma

Stromal FN has been found to be upregulated in HNSCC (Al Moustafa *et al.* 2002). In OSCC the presence and amount of stromal FN correlates with an increase in cervical metastases and extracapsular spread. More importantly, stromal FN has been found to be linked to increased disease-specific mortality in OSCC (Lyons *et al.* 2001). Additionally, a recent study showed that cancer cell intracellular FN is a predictive marker of delayed lymph node metastasis in OSCC (Luksic & Suton 2017).

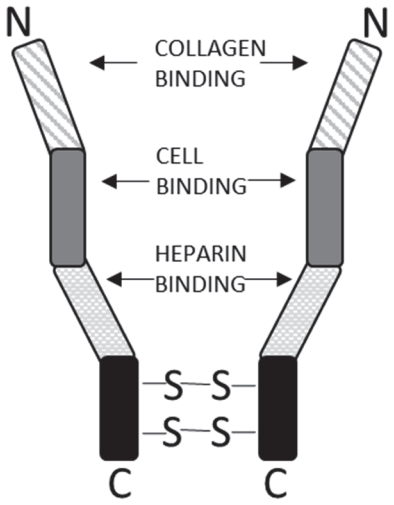

Fig. 4. Schematic illustration of the structure of FN.

3 Aims of the present study

The main aim of this study was to elucidate the role of various TME components in OTSCC invasion and prognosis. The more specific aims were:

1. To study the effect of hypoxia on carcinoma cell migration and invasion (I).
2. To study the effect of ECM and soluble factors from the TME on carcinoma cell migration and invasion (I, II).
3. To evaluate the prognostic significance of cancer cell intracellular and stromal TNC and FN in early-stage OTSCC (III).

4 Materials and methods

Table 5. Methods used in the original publications.

Level	Method	Publication
Cell experiments	Hypoxia experiments	I
	Migration assays	I, II
	Vertical migration	I
	Horizontal migration	I, II
	Invasion assays with tissue discs	I, II
	Native myoma (human)	I, II
	Rinsed myoma (human)	I, II
	Lyophilized myoma (human)	II
	Native heart (human, mouse, rat and porcine)	II
	Rinsed heart (human)	II
	Tongue (porcine)	II
Protein analyses	Western blot	I, III
	Gel filtration chromatography	II
	Mass spectrometry	II
	Radioimmunoassay	I, II
	Immunohistochemistry	I, II, III
	Manufacturing and characterization of antibodies	III
Other	Evaluation of intracellular and stromal TNC and FN	III
	Statistical analyses	I, II, III

Table 6. Antibodies used in the original publications.

Antibody	Clone	Source	Manufacturer's code	Dilution	Epitope retrieval	Publication
Cytokeratin	AE1/AE3	Dako, Denmark	M3515	1:250 or 150	Pepsin	I, II
Cytokeratin	Polyclonal	Novus Biologicals, UK	NB600-579	1:100	Pepsin	I
CAIX	Polyclonal	Santa Cruz, USA	(H120):sc-25599	1:50	Pepsin	I
TNC	DB7	Biohit, Finland	610003	1:500	Tris/EDTA	II, III
TNC	D2	Self-manufactured	D2	1:500	Tris/EDTA	III
S100	Polyclonal	Dako, Denmark	Z0311	1:3000	Pepsin	II
FN	568	Leica Biosystems, UK	NCL-FIB	1:500	Tris/EDTA	III
FN	F12	Self-manufactured	F12	1:500	Tris/EDTA	III
α-SMA	1A4	Dako, Denmark	M0851	1:500	Citrate	III

4.1 Patients and biological material (III)

OTSCC samples were used in order to study the prognostic significance of stromal and cancer cell intracellular TNC and FN. A total of 178 samples were obtained from OTSCC patients treated in the University Hospitals of Oulu and Kuopio in Finland, the Study and Treatment Cancer Center of Western Paraná and the Oncology Center of Cascavel in Brazil between 1979 and 2009. Ninety-eight patients had early-stage (clinical stage I – II; cT1 – T2N0M0) OTSCC. Only cases with adequate clinical information and biological material were included in this study. All patients were treated with primary resection of the tumor. The detailed patient demographic is shown in Table 1 in III.

4.2 Cell experiments (I, II)

4.2.1 Cell lines (I, II)

Malignant cell lines

Cell lines were used as monocultures (I), and in migration (I, II) and invasion (I, II) assays. An aggressive OTSCC cell line, HSC-3 (JCRB 0623; Osaka National Institute of Health Sciences, Osaka, Japan); OSCC cells isolated from primary (UT-SCC-43A) and recurrent (UT-SCC-43B) carcinomas of mandibular gingiva tumors; malignant melanoma cells G-361 (ATCC® CRL-1424™, Boras, Sweden) and breast adenocarcinoma cells MDA-MB-231 (ATCC® HTB-26™, Boras, Sweden) from a metastatic site were used in this study.

Other cell lines

CAFs (CaDEC12) that had been isolated from a specimen of OTSCC (Dayan *et al*. 2012) and normal gingival fibroblasts (NOF) that were isolated from normal superfluous buccal mucosa tissue after wisdom tooth extraction (Costea *et al*. 2005) were used in co-cultures with HSC-3 cells (II). The cell culture protocols are described in original publications I (HSC-3, UT-SCC-43A and 43B) and II (CAF, NOF, G-361 and MDA-MB-231).

4.2.2 Hypoxia experiments (I)

Two different methods were applied in order to induce hypoxic stress in cells: the experiment was either performed in a hypoxia chamber or the cells were exposed to $CoCl_2$. In invasion assays, the myoma discs with cells were placed in the hypoxia chamber (InVivo2 400, Ruskinn Technology, UK) and exposed to 1% oxygen for 20 h on day 1 after placing the myomas onto the steel grids. Cell cultures for western blot analysis were cultivated in the hypoxia chamber for 20 h, after which they were lysed immediately. In the horizontal migration assay, the cells were cultivated under hypoxic atmosphere starting from the scratching until the fixation after 0 h, 16 h, or 48 h (I); or 20 h (II).

$CoCl_2$ prevents the degradation of HIF-1α and thus mimics the effect of hypoxia through the HIF-1 pathway (Brusevold *et al.* 2010, Carroll & Ashcroft 2005, Epstein *et al.* 2001, Yuan *et al.* 2003). A concentration of 100μM was used in the experiments. The myoma invasion assay was modified according to Brusevold *et al*. (2010). The cells were exposed to $CoCl_2$ overnight after they were allowed to attach to the myoma for one day.

4.2.3 Migration assays (I, II)

Vertical migration (I)

A Transwell migration assay was performed to determine the most aggressive oral cancer cell line among those used in this study (I). The vertical migration activity of HSC-3, as well as UT-SCC-43A and -43B cells was studied using Transwell inserts (Corning, NY). 30,000 cells suspended in serum-free media were added in each of the inserts on top of the membrane. The cells were allowed to migrate for 20 h, after which they were fixed with trichloroacetic acid (TCA). The cells were stained with crystal violet and the results were quantified visually in six different fields around the center of the membrane from each sample using a microscope (Leica, Germany). The results are presented as number of migrated cells.

Horizontal migration (I, II)

Wound healing assays were performed to study the effect of hypoxia (I: performed in normoxia, hypoxia and with $CoCl_2$ treatment) and soluble fragments from myoma and heart tissue (II) on cancer cell migration. For the horizontal migration

assay, the cells (HSC-3, UT-SCC-43A and -43B in I; HSC-3 in II) were grown to confluency on chamber slides (Corning) and wounds were created using a 1 ml pipette tip. Wound closure was monitored at different time points. The cultures were fixed with TCA after 0 h, 16 h or 48 h (I), or after 20 h (II), and stained with crystal violet. Scratch areas of samples from different time points were compared to the 0 h samples, and proportional scratch areas were calculated. Images were captured and color recognition (MCID Core 7.0 image analysis software; Nikon, Japan) was utilized to measure the cell density of stained cells in each field. The proportional scratch area for each scratch was measured as follows: proportional scratch area = 1 - (cell density in scratched area)/(cell density in unscratched area). We performed a control measurement of the cell density in the unscratched area in order to normalize the effect of cell proliferation on the wound healing rate, hence adding sensitivity to the final horizontal migration analysis.

4.2.4 Invasion assays (I, II)

Invasion assays were performed to study the effects of hypoxia (I), soluble factors (I, II), lyophilization (II) and non-neoplastic microenvironments (II) on cancer cell invasion. To study the invasion activities of various cancer cells, the human uterine leiomyoma organotypic assay developed by our group (Nurmenniemi *et al.* 2009) was utilized. Porcine tongue, and human, porcine, mouse and rat hearts were used as non-neoplastic microenvironments (II).

Myoma organotypic assay (I, II)

Human uterine leiomyoma resections were obtained from otherwise healthy patients in routine surgery in Oulu University hospital after the patients provided an informed consent. Myoma samples were cut into 3 mm thick slices and discs were formed using an 8 mm biopsy punch. Only the non-degenerated solid myomas were used and myomas were tested for their invasion aiding properties using the highly invasive HSC-3 cell line prior to the experiments. Approximately 100 myoma discs were obtained from each myoma, and discs from the same myoma were used in each individual experiment. The invasion assays were performed as described by Nurmenniemi *et al.* (2009). Briefly, the discs were inserted into Transwell inserts and the HSC-3 (I, II), UT-SCC-43A and -43B (I), MDA-MB-231 (II) or G-361 cells (II) [700,000 alone; or 200,000 in co-cultures with fibroblasts (CAFs or NOFs) (II)] were seeded on top of each disc. The cells were allowed to

invade for 11 – 14 days. Radioimmunoassays (RIA) were performed on culture media to detect type III collagen degradation fragments as described in original publications I and II. After the incubation period the discs were prepared for histology and immunohistochemistry.

Maximum invasion depth and the area of invaded cells were measured from myoma samples. QWin V3 software (Leica Microsystems) was utilized in the analysis of invasion depth and area. Each sample was digitally recorded using 100x magnification. From each sample, the invasion depth was measured as the distance of the deepest invading cell from the closest part of the dormant, non-invading, cell layer on top of the tissue disc. Color recognition was used in the analysis of invasion area: from each acquired picture of the tissue sample, the color of immunostained cells was detected and the total area of those detected cells measured. Next, the area of the non-invading cells was defined and subtracted from the total area to determine the area of invading cells.

Myoma invasion assays were additionally performed using rinsed (I) and lyophilized (II) myoma discs to study whether myoma tissue maintained its invasion aiding properties after these treatments. Moreover, the effect of soluble factors of myoma tissue was studied by comparing invasion assays performed using native and rinsed myomas. Myoma rinsing medium (RM) was used as a substrate in tongue invasion assays and wound healing assays (II). The rinsing process is described in detail in original publication I and the lyophilization process in original publication II.

Heart invasion assay (II)

Human, porcine, mouse and rat hearts were used in heart invasion assays. The human heart sample was a gift from Semmelweis University, Budapest, Hungary. The porcine heart was collected from a blood donor animal from the Laboratory Animal Centre, University of Oulu. Human and porcine heart discs were prepared similarly to the myoma organotypic model.

Mouse and rat hearts were collected from healthy animals from the Laboratory Animal Centre, University of Oulu. Tissue discs were prepared similarly to myoma discs with the exception of size: the diameter of mouse and rat heart discs was 3 mm. Invasion assays were performed by placing 100,000 cells (HSC-3, MDA-MB-231 or G-361) in serum-free media on top of the heart tissue discs. The heart organotypic cultures were carried out similarly to myoma invasion assays during the 14-day experiment.

Porcine tongue invasion assays (II)

The tongues from healthy pigs were collected from the Laboratory Animal Centre, University of Oulu, from animals that had been used by other group (Mäkelä *et al.* 2013). The development of the tongue model is described in original publication II. After preparation, the discs were transferred into Transwell inserts and the cells (HSC-3 with or without CAFs or NOFs, 200,000 in serum-free media) were added on top of the discs. The tongue invasion assays were sustained for 9 – 11 days and the media were changed daily.

Invasion assay with HSC-3 and CAFs or NOFs (II)

CAFs were used in co-culture with HSC-3 cells in an attempt to facilitate invasion in tongue discs. NOFs were used as control cells. 200,000 HSC-3 cells were added on top of myoma or tongue discs, with or without CAFs or NOFs (200,000). All the cells were fluorescently labeled, HSC-3 cells with green (DiO) and CAFs/NOFs with red dye (Dil) (Vybrant labels, Invitrogen). Normal medium or myoma RM was used in those cultures utilizing tongue discs, and only normal medium for those with myoma discs. The invasion assays were maintained for 11 days. DAPI staining was used and the stained sections were photographed using an AMG EVOSfl microscope system.

4.3 Western blot (I, III)

Western blot was utilized to detect production of CAIX in HSC-3 cells in monolayer culture (I). Additionally, western blot was utilized in characterizing the target antigens of our novel mouse anti-human antibodies (III). Full details of the western blots are described in the original publications I and III.

4.4 Gel filtration chromatography (II)

Gel filtration chromatography was used to separate the fractions of myoma and heart RM. Three adjacent fractions from each RM were pooled and added to culture media separately, and the effect of the fraction pools on cell migration was studied using wound healing assays as described above. The filtration procedure is described in detail in original publication II.

4.5 Mass spectrometry (II)

The proteome of myoma rinsing media was studied using mass spectrometry. Myoma discs were rinsed for 24 h and the rinsing media were combined into two pools and concentrated. Similar mass spectrometry proteome analysis was performed for both pools. The full details of mass spectrometry are given in original publication II.

4.6 Radioimmunoassay (RIA) (I, II)

A type III collagen C-terminal peptide (IIICTP) radioimmunoassay was utilized in order to investigate whether tissue disc ECM invading cancer cells were degrading type III collagen. Polyclonal antibodies against the carboxy terminal region of type III collagen were raised in rabbits (Nurmenniemi *et al*. 2009, Nurmenniemi *et al*. 2012). Synthetic peptide 99 was used as a IIICTP antigen. The IIICTP RIA was conducted as described in original publications I and II.

4.7 Immunohistochemistry (I, II, III)

Immunohistochemical stainings were used in all of the original publications. Immunohistochemistry was utilized to detect the following: invaded cells in tissue discs (I, II), cells under hypoxic stress in myoma tissue (I), the expression of TNC in myoma tissue (II) and the expression of α-SMA, TNC and FN in cancer cells and in tumor stroma (III). The precise protocols for immunohistochemistry are described in the original publications, and the antibodies and pre-treatments used are summarized in Table 6. Most of the antibodies used were commercially available, and two (D2 for TNC and F12 for FN) were manufactured by our collaborators.

4.7.1 Manufacturing and characterization of antibodies (III)

Monoclonal anti-human antibodies were manufactured by immunizing mice with human mesenchymal stromal cells (hMSCs) that were obtained from bone marrow of two volunteers with arthritis. This was a part of a larger project by our collaborators (Prof. Lehenkari's group). Human cells were used in order to obtain highly specific antibodies to be used in research conducted using human cells and tissues. Immunization of mice led to the production of antibodies against several

different proteins. These antibodies were characterized and their target proteins identified. Two of the hybridoma cell lines (A8 and D2) produced antibodies against TNC and three (F12, B6 and C1) against FN. Additionally, several more target antigens were identified. We selected the antibodies from clones D2 and F12, compared them to commercial antibodies (DB7 for TNC and NCL-FIB for FN) and used them to stain OTSCC samples in order to study the prognostic value of stromal and cancer cell intracellular TNC and FN in OTSCC with special emphasis on early-stage cases. The immunization of the mice, and antibody isolation and characterization are described in original publication III.

4.7.2 Evaluation of intracellular and stromal TNC and FN (III)

Stromal and cancer cell intracellular staining for TNC and FN were analyzed from OTSCC sections to evaluate their prognostic significance. Stromal staining for TNC and FN was scored as described by Bello *et al.* (2011) using a scale from 0 to 4. For statistical analysis the score 0 was labeled as "negative", grades 1 – 3 were merged into the same "moderate" group and grade 4 was labeled as "abundant" (Fig 5). All the tumors were scored according to the most severe grade if areas of different grades were detected.

Intracellular staining in carcinoma cells was scored from 0 to 2. For statistical analysis the groups 1 and 2 were merged into a combined "positive" group (Fig 6). Scoring was performed independently by two of the authors who were unaware of the clinical outcome at the time of the analysis. Discrepancies in the scoring were settled by a third person. A more detailed description of stromal and intracellular scoring criteria can be found in original publication III.

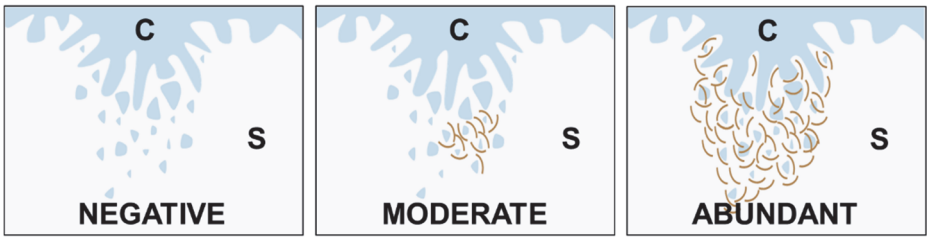

Fig. 5. Staining evaluation of stromal TNC and FN. C = cancers cells, S = stroma.

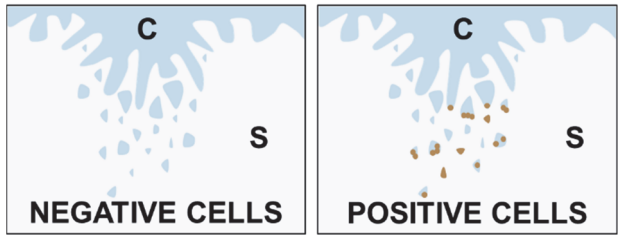

Fig. 6. Staining evaluation of cancer cell intracellular TNC and FN. C = cancers cells, S = stroma.

4.8 Statistical analysis (I, II, III)

When analyzing the results from migration assays, invasion assays and RIA (I, II), statistics were evaluated using a two-sample t-test. *P*-values ≤ 0.05 were considered statistically significant.

Cumulative survival curves of patients in stromal and intracellular TNC and FN analyses were created using the Kaplan-Meier (KM) method and disease-specific death as the endpoint. Censoring events were death by any cause other than cancer and the end of follow-up. Differences between groups were evaluated using a log-rank test, and *p*-values ≤ 0.05 were considered statistically significant. Analyses were performed for a pooled group including all the stages of OTSCC from all hospitals, as well as for early-stage cases alone.

Uni- and multivariate analyses were conducted using a Cox proportional hazard model to determine if stromal or intracellular TNC or FN is an independent prognostic indicator. In multivariate analysis the hazard ratios (HRs) for TNC and FN were adjusted for age, gender, hospital, adjuvant therapy and clinical stage (among the pooled group of all the stages).

Correlations between stromal TNC, FN and α-SMA were calculated using the Spearman correlation and 2-tailed significance was analyzed.

The analysis of migration assays, invasion assays, RIA (I, II) and KM analysis (III) were performed using OriginPro 2015 (OriginLab, Northampton, MA, USA), and the Cox proportional hazard model (III) using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

4.9 Ethical considerations (I, II, III)

The use of myoma (I, II) was approved by the Ethics Committee of the Oulu University Hospital (EETTMK: 35/2014). Collection of human heart samples (II) was approved by the Health and Scientific Research Ethical Committee in Budapest. Porcine, mouse and rat tissues (II) were collected from animals used by other research groups who had ethical permissions for the use of those animals from the National Animal Experiment Board (ELLA) or Research Animal Care and Use Committee of the University of Oulu. The use of OTSCC samples (III) was approved by National Supervisory Authority for Welfare and Health (Valvira, Dnro 6865/05.01.00.06/2010). In Brazil, this study was approved by the Ethics Committee in Research of the Piracicaba Dental School, University of Campinas (approval number 100/2012).

5 Results

5.1 The effect of hypoxia is dependent on the surrounding tissue and cell line (I)

Horizontal migration was studied using two OSCC cell lines (UT-SCC-43A and -43B) and one OTSCC cell line (HSC-3). Hypoxia increased the migration of all the cell lines used, but the effect was most prominent using HSC-3 cells. No effect was observed on migration when $CoCl_2$ treatment was used in comparison to normoxia (Fig 2 in I, Fig 7).

After the effect of hypoxia on cancer cell migration was confirmed and shown to be cell line dependent, the effect of hypoxia on cancer cell invasion was studied. The most aggressive of our cell lines, HSC-3, was used.

In native myoma, invasion depth was deepest in control myomas cultivated in normoxia; $CoCl_2$ treatment decreased the invasion area, whereas no difference was observed between control and hypoxia groups (Fig 7). In native myoma, no differences were observed in type III collagen degradation (Fig 4 in I).

In rinsed myoma, however, the effects of hypoxia and $CoCl_2$ were reversed: invasion depth was greater in hypoxia and $CoCl_2$ groups compared to normoxia, and invasion area was increased in the $CoCl_2$ group compared to normoxia (Fig 7). As in native myoma, no differences were detected in type III collagen degradation in rinsed myoma (Fig 4 in I).

These results show that in the analysis of different factors (hypoxia in this case) it is not reasonable to oversimplify the environment in which the experiments are conducted as the environment could have an impact on the effect of the factor under investigation.

5.2 The pattern and mechanism of invasion is dependent on the surrounding tissue (I, II)

When experiments were conducted in normoxia that tested the invasion of UT-SCC-43A, -43B and HSC-3 cells into native and rinsed myoma, 43B degraded more type III collagen compared to 43A (Fig 3 in I); this increased aggressiveness of cells from a secondary tumor could be expected based on the migration assay (Fig 1 in I). Type III collagen degradation was most abundant with HSC-3 cells. Interestingly, enzymatic type III collagen degradation was increased with all the cell lines in rinsed myoma (Fig 3 in I).

Additionally, we used lyophilized and rehydrated myoma discs in invasion assays alongside the native, non-lyophilized, discs. HSC-3 cells were used in this experiment. There was no difference in invasion depth between native and lyophilized myoma. However, there was less type III collagen degradation in lyophilized myoma compared to native discs (Fig 3 in II).

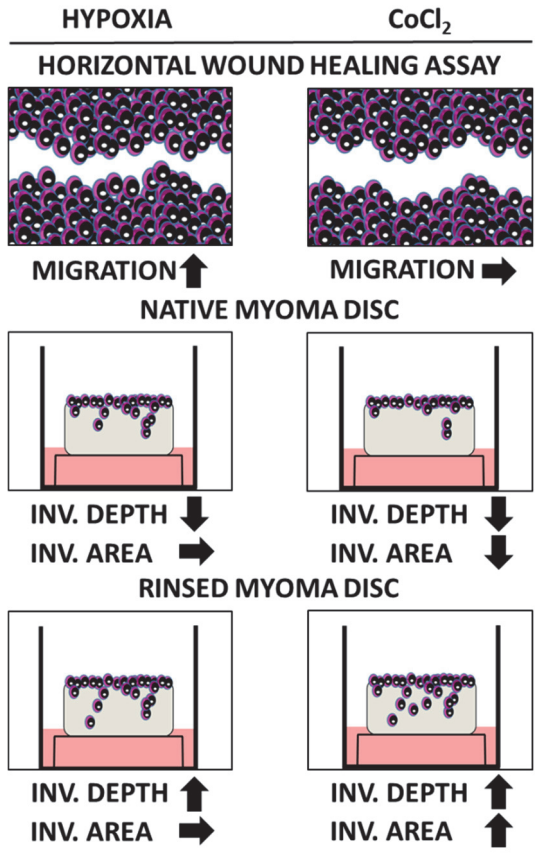

Fig. 7. The effect of hypoxia and $CoCl_2$ on HSC-3 migration and invasion when compared to normoxia. → = no change, ↑ = increase and ↓ = decrease.

5.3 Non-neoplastic tissue microenvironment does not support invasion of carcinoma cells (II)

In addition to modifying the myoma model, we studied whether non- neoplastic tissues can be utilized in cancer cell invasion studies similar to myoma. We tested human, porcine, mouse and rat heart tissue and porcine tongue tissue. Myoma tissue was used as a control. The human heart tissue disc experiment was performed using rinsed discs in addition to native discs. We utilized the highly invasive HSC-3 cells in these experiments to ensure that invasion would occur if possible. Additionally, MDA-MB-231 and G361 cells were used.

Only minimal invasion could be seen in human heart tissue (Fig 4 and 5 in II) and no invasion was detected in rat, mouse or porcine tongue or heart tissue (not shown). Rinsing the discs prior to the invasion assay slightly induced invasion of MDA-MB-231 and G-361 cells into human heart tissue (Fig 5 in II).

Since rinsing the myoma tissue resulted in increased degradation of type III collagen, and rinsing the heart tissue resulted in a slightly elevated level of invasion, we hypothesized that there could be cell motility affecting soluble factors in those rinsing media. We tested this hypothesis by using wound healing assays in which we added the lyophilized residues from RMs originating from myoma and heart tissues. Indeed, this resulted in decreased migration in those wounds where small molecular weight fractions from heart RM were used when compared to myoma RM (Fig 6 in II).

Next we decided to elucidate further what this cell migration increasing myoma RM contained. Mass spectrometry was utilized to analyze the proteome of myoma RM. This revealed a variety of growth factors as well as their receptors and binding proteins (Table 1 in II). Indeed, some of these are known to increase cell migration *e.g.* fibroblast growth factor 2 (FGF2), hepatocyte growth factor (HGF) and transforming growth factor beta (TGF-β) 1 and -2.

Table 7. Multivariate analysis of stromal TNC and FN.

All stages	HR	95% CI for HR
Stromal TNC		
Negative	1	
Moderate	1.14	0.60 - 2.17
Abundant	2.48	1.04 - 5.90
Stromal FN		
Negative	1	
Moderate	5.33	2.07 - 13.73
Abundant	9.64	3.34 - 27.83
Early-stages	HR	95% CI for HR
Stromal TNC		
Negative	1	
Moderate	0.76	0.23 - 2.48
Abundant	6.54	1.20 - 35.75
Stromal FN*		
Moderate	1	
Abundant	5.91	1.75 - 19.94

*HR could not be calculated compared to "Negative" group due to lack of end-points in that group.

5.4 Stromal TNC and FN are prognostic indicators of OTSCC (III)

Since there is variation in invasion efficiencies between myomas from different patients, we investigated if there is variation in the ECM of these myomas. TNC content was chosen as the variable of interest in the ECM. Indeed, there were remarkable differences in TNC staining between different myomas (Fig 3 in II). After a difference in the TNC content in myomas was confirmed, we stained OTSCC sections with a TNC antibody to test whether it may have value in prognostication. In addition to TNC we chose FN to be used in prognostication as well; FN is a protein that is present in the TME of many cancers and has been used previously as a prognosticator. We stained a total of 178 OTSCC samples using monoclonal antibodies for TNC and FN and analyzed the stromal and cancer cell intracellular expression of these proteins.

In the pooled group including all the stages of OTSCC, the stromal TNC was found to be a prognosticator when detected abundantly. In this group, also FN was found to be a prognosticator with moderate and abundant staining, both indicating gradually worsening prognosis (Table 7). The intracellular TNC and FN expressions in cancer cells, however, were not as reliable prognosticators as those in stroma.

5.5 Stromal TNC and especially FN are prognosticators of early-stage OTSCC (III)

When TNC and FN were proven to be reliable prognosticators in a pooled group of all the stages of OTSCC, we analyzed the early-stage cases separately. As was the case in all the stages, abundant stromal TNC was a prognosticator among early-stages. Surprisingly, stromal FN was a highly accurate prognosticator since there were no cancer deaths in the stromal FN negative group. Additionally, the abundant group had significantly poorer prognosis compared to that of moderate group (Table 7).

5.6 Abundant stromal TNC and FN predict unfavorable outcome (III)

Since both TNC and FN were found to have prognostic value, we analyzed the correlation of their expression in tissue samples. Both stromal TNC and FN were expressed in the fibrillar network of the ECM and in fibroblast-like cells. The expressions of TNC and FN were found to be correlated in all stages and early-stages. Only a weak correlation was found between stromal α-SMA and TNC or FN.

When both stromal TNC and FN were found to be prognosticators individually and their expression to be correlated, we analyzed the outcomes of those cases that had abundant staining of both TNC and FN in the tumor stroma. The analysis of those patients with tumors that had abundant stromal staining of both TNC and FN revealed that among all stages there was only one patient alive after follow-up and none in early-stage cases.

6 Discussion

6.1 Prognostic significance of TME

As the role of TME in cancer progression has become abundantly clear, it is a logical continuum to search the TME for prognostic markers. These prognosticators can be related to cells, ECM or soluble factors in the TME. Putative prognosticators have been found from each one of these subsets in various cancers.

Here we have shown that alterations in each of these factors affect the invasion efficiency and mechanism of cancer cells. There were significant variations in invasion activity between different OSCC cells (I) and invasion has been previously shown to increase in the presence of mesenchymal stem cells (Salo *et al*. 2013). Invasion efficiency was altered when soluble factors were removed from tissue discs (I) and alteration of ECM structure by lyophilizing resulted in a switch in invasion mechanism (II). Additionally, invasion was impaired in a non-neoplastic tissue environment (II). More importantly, we demonstrated that in *in vitro* cancer studies it is crucial to use a microenvironment that resembles the natural environment of the cancer as closely as possible.

6.2 Myoma mimics TME

When invasion experiments were conducted utilizing the myoma invasion assay, the deepest invasion was detected in intact myoma in normoxic conditions. The same level of invasion was achieved in rinsed myoma when the experiment was conducted under hypoxia or $CoCl_2$ treatment. Additionally, CAIX was detected in invaded HSC-3 cells in myoma tissue even under normoxia. Taken together, it is reasonable to deduce that myoma tissue itself provides a hypoxic microenvironment with a gradient of hypoxia similar to the *in vivo* situation (I). Indeed, this has been suggested in a previous study by Mayer *et al.* (2008). Additionally, Mehine *et al.* (2013) have shown that myoma tissue has an altered genetic profile similar to that of malignant tumors; this may facilitate the progression of myoma into a more "invasion friendly" environment when compared to non-neoplastic tissue.

$CoCl_2$ mimics hypoxia via disruption of HIF-1 and -2 pathways and is comparatively easy to use. However, since the effect of $CoCl_2$ is based on HIF-1 and -2 routes, it does not sufficiently mimic hypoxia as we demonstrated using immunoblotting for CAIX (I). Additionally, in rinsed myoma tissue, both $CoCl_2$

treatment and hypoxia increased the invasion depth to the same higher level, but in intact myoma this increase in invasion depth was not seen. This can be hypothesized to be due to depletion of hypoxia induced factors in the soluble component of TME in rinsed tissue; in intact tissue the environment itself contains all the factors needed for increased invasion. To support this, there have been other studies, as early as in the 1960s, in which tumor interstitial fluid (TIF) was analyzed. These studies found that TIF has high H^+, CO_2, and lactic acid and low glucose and O_2 compared to afferent plasma (Gullino *et al.* 1964, Gullino 1966). These findings, especially high lactic acid and H^+, suggest high activity of anaerobic metabolism caused by hypoxia. More recent studies have found that TIF contains a fraction of small molecular weight (<25 kDa) molecules that are distinct from plasma (Stohrer *et al.* 2000, Wiig *et al.* 2003). Moreover, we found in our study (II) that a small molecular weight fraction from myoma RM (=TIF) induced migration of OTSCC cells. In both cases – previous studies and our study – those small molecular weight molecules are yet to be identified, thus creating an avenue for further research.

The myriad signaling pathways and their interactions in living organism are too complex to be simplified to bare minimums – as is often done in *in vitro* experiments – without affecting the end result. For example even as simple a factor as lack of oxygen is not straightforward to mimic by impairing only one signaling route. It is crucial that in functional assays with various cell lines the environment in which the experiments are conducted should be kept as close as possible to that found *in vivo*, since signals originating from the microenvironment may affect the cell's response to the factor under investigation.

Apart from providing a hypoxic microenvironment, myoma tissue provides an environment resembling TME including ECM. When myoma discs were stained using monoclonal TNC antibody there was great variation in the amount of TNC between different myoma discs (II). Furthermore, we noticed that invasion depth was greater in those discs with more TNC. This led to the idea of studying the prognostic value of TNC in OTSCC. Since TNC is often associated with FN in prognostication, as well as in cancer cell dissemination and invasion, we also used FN in prognostication in addition to TNC.

6.3 Prognostic significance of TNC and FN (III)

The two ECM proteins analyzed in this study, TNC and FN, have been previously studied and found to be prognosticators or indicators of a more aggressive phenotype in several cancers *e.g.* breast, colorectal, and esophageal carcinomas

among others (III). However, to our best knowledge this is the first study to show their prognostic significance in early-stage OTSCC.

TNC is a matricellular ECM protein, which regulates cell proliferation, adhesion and migration (Chiquet-Ehrismann & Chiquet 2003). During carcinogenesis TNC is involved in several hallmarks of cancer, including cell proliferation and survival, angiogenesis, stromal cell recruitment and immunomodulation, cell migration and invasion, organ tropism, and it promotes a stem cell like state and therapy resistance (Thakur & Mishra 2016). In OSCC, stromal TNC has previously been found to be deposited in the invasive front of the tumor (Hindermann *et al.* 1999), and to be increased in quantity alongside the degree of dysplasia of oral epithelium (Tiitta *et al.* 1994). Despite being under vigorous investigation for several decades, we found no studies in which TNC was studied immunohistochemically for its prognostic significance in early-stage OTSCC.

In our study (III), the expression of stromal TNC predicted disease-specific mortality of early-stage OTSCC patients, especially when abundant staining was detected. However, the cancer cells' intracellular TNC failed to provide prognostic value. This can be hypothesized to be due to the nature of TNC's role as an ECM protein: it cannot affect the cellular functions from within the cells where it cannot bind to receptors and growth factors, or to soluble and ECM bound proteins through which TNC's effects on cellular functions are mediated. This hypothesis is supported by a review by (Murphy-Ullrich & Sage 2014) in which they pointed out that there are intracellular roles for some matricellular proteins but TNC was not listed as one of those.

FN is a structural, multifunctional ECM glycoprotein that is organized in a fibrillary network. FN regulates *e.g.* cellular growth, differentiation, adhesion and migration (White *et al.* 2008). During carcinogenesis FN is involved in invasion and metastasis, and additionally it is a marker of EMT and angiogenesis (Chong *et al*. 2012, Rybak *et al*. 2007, Stivarou & Patsavoudi 2015). Furthermore, stromal FN correlates with poor prognosis, aggressive phenotype or other features of poor prognosis in various cancers, including OSCC (Al Moustafa *et al.* 2002, Ferrari *et al.* 2009, Ioachim *et al.* 2002, Ioachim *et al.* 2005, Lyons *et al.* 2001, O'Shannessy *et al.* 2014). However, to our best knowledge, there were no immunohistochemical studies of the role of FN as prognosticator for early-stage OTSCC.

In our study, stromal FN was shown to be an excellent prognosticator in all stages of OTSCC, and more importantly among early-stage cases; in early-stage cases 100% of those with negative stromal FN staining were alive after a 5-year

follow-up. Our results are in concordance with previous studies on the predictive value of stromal FN. The main ligands through which FN mediates and coordinates the interactions between ECM and cells are integrins. FN has been suggested to affect cancer invasion and metastasis through activation of the PI3K/Akt pathway resulting in up-regulation of pro-invasive MMPs -2 and -9 (Das *et al*. 2008, Maity *et al*. 2011). Additionally, in their review Kaplan *et al.* (2006) pointed out that newly formed FN has a pivotal role in preparing the "soil" of the premetastatic niche prior to attachment of circulating cancer cells.

In addition to being independent prognosticators, TNC and FN had prognostic value in combination; when both stromal TNC and FN were abundant, there were no patients alive after a 5-year follow-up among early-stage patients. Hence, both extremes – negative and abundant – of stromal stainings can be of clinical importance: negative stromal FN is an indicator of less aggressive disease, indicating that primary tumor resection and close monitoring should be favored as opposed to resection with neck dissection and possible adjuvant therapy. However, if both stromal TNC and FN are abundantly stained, it should be interpreted as a strong indication for neck dissection and multimodality treatments.

The synergistic role of TNC and FN in carcinogenesis and invasion has been studied previously and they both have been suggested to be crucial for the dissemination and invasion process as antagonists for each other, TNC being anti- and FN pro-adhesive (Yoshida *et al.* 1997). TNC has been shown to inhibit cell adhesion to FN and thus FN-mediated cell migration through binding directly to FN, as well as binding to syndecan-4, a transmembrane heparan sulfate proteoglycan that acts as a coreceptor for integrin α5β1, which is required for full spreading of cells on FN (Huang *et al.* 2001, Salmivirta *et al.* 1991). By competing with FN in binding to syndecan-4, TNC additionally inhibits cell proliferation via inhibition of RhoA, FAK and tropomyocin-1 activity (Lange *et al.* 2008).

6.4 Shortcomings of the study

As science is only improved by learning from mistakes and shortcomings, it is imperative to recognize those not only in the work of others, but more importantly in one's own work. Here are listed and discussed the shortcomings of the original publications that form the base of this thesis as they are viewed by the author in retrospective.

We showed that the effect of hypoxia is also mediated through pathways other than the well-established HIF-1 and -2 pathways because $CoCl_2$, which inhibits the

HIF-1 and -2 pathways, did not inhibit the expression of the hypoxic marker CAIX in cell culture. In this study we did not do a control to establish that the HIF-1 and -2 pathways were in fact blocked when using $CoCl_2$.

In study I we showed that in rinsed myoma tissue collagen degradation was induced, but invasion depth diminished in comparison to native, unrinsed myoma tissue. Additionally, in study II we were able to show that soluble factors from heart tissue inhibited the migration of OTSCC cells when compared to those from myoma tissue. Furthermore, we analyzed the rinsing media from myoma tissue and found a variety of growth factors as well as their binding proteins and receptors. However, aside from this characterization we did not show the key players nor the effect of the variation in the amounts of these molecules on cancer cell migration and invasion, and their effect can only be deduced from previous literature.

When the prognostic value of TNC and FN were studied, the stainings were performed using only one antibody. Although the stainings were qualitatively compared to those performed using commercial antibodies, this has to be mentioned as a shortcoming since performing the scoring of the samples in duplicate using another antibody would have added to the rigor of this method. Additionally, the number of patients was unfortunately not optimal: *e.g.* in the FN abundant group there were only 7 early-stage cases.

6.5 Clinical implications

The most prominent part of this study from a clinical point of view is the prognostic value of TNC and FN. In the original publication III we found that stromal TNC and especially FN can be utilized in prognostication of early-stage OTSCC: the absence of FN in the tumor stroma is a strong indicator to withhold from neck dissection and multimodality treatments. On the other hand, if both stromal TNC and FN are abundant, it is highly recommended to consider elective neck dissection and multimodality treatments.

Furthermore, the use of intact myoma provides an ideal 3D *in vitro* invasion assay to study *e.g.* drugs, irradiation and other factors related to cancer therapy and cellular response in addition to invasion activity and mechanism pre-clinically with no need for animal-based assays. Theoretically, this could be performed using cancer cells isolated from patients with cancer, thus acting as a stepping stone towards more personalized medicine.

6.6 Topics for further research

As established here, the TME has a crucial impact on the behavior of cancer cells and this should be accounted for both in basic research as well as in diagnosis and prognostication.

In original publications I and II we modified tissue discs by removing soluble factors from their ECM by rinsing them. Furthermore, we analyzed the effect of this deprivation of soluble factors on cancer cell invasion, as well as the effect of these factors on cancer cell migration. Finally, we analyzed the proteome of migration and invasion increasing myoma RM. However, we did not study the effect of different relative amounts of these molecules. Keeping in mind the complex interactions and effects of different environmental factors it is unlikely that a single factor is the key to enhancing or inhibiting invasion. The relative distribution of several factors, however, may regulate this cellular behavior. Thus the distinction between a pro- and anti-invasive milieu would be an important avenue of further research related to the soluble component of the TME.

Additionally, the prognostic value of both stromal TNC and FN was established individually, and more notably, together. We analyzed TNC and FN from the TME of surgically removed tumors and the analysis pinpoints those individuals that would benefit from elective neck dissection. However, when these proteins are analyzed from tissue sections, the immunohistochemistry takes days to complete. The optimal situation would be to be able to perform the elective neck dissection simultaneously to primary tumor resection in order to avoid multiple surgeries. This could be accomplished if the analysis of TNC and FN could be performed from pre-surgical biopsies. Hence, a straightforward topic for further research is: can stromal TNC and FN be analyzed from biopsies with the same accuracy as from resected tumors? Additionally, after confirming the prognostic role of TNC and FN retrospectively with a larger patient cohort, a prospective study should be conducted using them in clinical decision making.

Almangush *et al.* (2015) have shown the BD-model to be an accurate prognosticator of early-stage OTSCC. Additionally, the BD-model has been tested using pre-surgical biopsies (Almangush *et al.* 2017). The BD-model concentrates on the analysis of cancer cells, whereas the analysis of stromal TNC and FN excludes the cancer cells. Presuming that the composition of tumor stroma affects the behavior of tumor cells (as is quite comprehensively shown), these two models should correlate with each other. However, it should be studied if these two models have synergistic prognostic value and ultimately, what are the common

denominators for the formation of cancer cell budding and deep invasion, and accumulation of TNC and FN in tumor stroma.

7 Summary and conclusions

As demonstrated, hypoxia and the soluble factors of the TME affect the behavior of cancer cells, including migration and invasion, the first steps towards metastasis. On the other hand, cancer and stromal cells alter their environment and thus form feedback loops of mutual alterations between cells and their environment in addition to cell – cell interactions. As there seems to be a delicate balance in the interactions concerning cells and the TME in cancer progression, any slight change (*e.g.* change in proportions of growth factors) in this complex signaling network can tip the scale, for example from a cancer invasion hindering TME to an invasion aiding one. This complexity causes conditions present in *in vitro* studies of cancer cell behavior to differ significantly from the environment in which cancer cells interact *in viv*o, especially when cells are cultured merely on plastic, as there are no external stimuli with which cancer cells could interact as in their natural environment. Different animal-based materials have been developed to bridge this gap between plastic and the actual TME. However, as shown here, tissues of different species, especially non-neoplastic tissues, do not properly mimic human TME. Hence the need arises for a human TME mimicking substance, which myoma tissue provides.

It is obvious that TME has a great influence on the prognosis of cancers through its ability to first act as a barrier for cancer cells, and further down the line to be an accomplice rather than bystander in invasion and metastasis. This has led to many investigations for prognostic TME markers, and many have been found. Unfortunately, novelty has been prioritized over confirmations of previous findings in the search for these markers and many have been published only once, and this has not led to adoption of these markers as tools of routine pathology. Here we analyzed the prognostic significance of two well studied ECM proteins: TNC and FN. Of these two, especially TNC has been a subject of rigorous research in terms of carcinogenesis and over a decade ago was predicted to be an important prognosticator. In some cancers it has prognostic significance, as reviewed above. Surprisingly, not many publications were related to TNC in the ECM of OSCC and fewer in OTSCC. The most important finding here related to TNC and FN was their unambiguous role in the stroma of early-stage OTSCCs: if both TNC and FN were abundant, 100% of those patients died during follow-up. However, if stromal FN was negative, none of those patients died of OTSCC. This should give a very easily interpreted signal to withdraw or utilize the most aggressive treatments in these cases.

Altogether, this study underlines the pivotal role of the TME in cancer progression and prognostication, highlights the importance of adequate methods in *in vitro* studies, as well as provides tools for prognostication of early-stage OTSCC, and creates avenues for further research for better prognosticators and possible targets for medical intervention.

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Original publications

I Teppo S, Sundquist E, Vered M, Holappa H, Parkkiseniemi J, Rinaldi T, Lehenkari P, Grenman R, Dayan D, Risteli J, Salo T* and Nyberg P* (2013) The hypoxic tumor microenvironment regulates invasion of aggressive oral carcinoma cells. Exp Cell Res 319(4): 376-389

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* Equal contribution to the supervision of this study.

Original publications are not included in the electronic version of the dissertation.

1426. Karhu, Toni (2017) Isolation of novel ligands for MAS-related G protein-coupled receptors X1 and X2, and their effect on mast cell degranulation

1427. Mantere, Tuomo (2017) DNA damage response gene mutations and inherited susceptibility to breast cancer

1428. Salokorpi, Niina (2017) Treatment of craniosynostoses

1429. Männikkö, Niko (2017) Problematic gaming behavior among adolescents and young adults : relationship between gaming behavior and health

1430. Kortekangas, Tero (2017) The non-operative treatment of Weber B -type ankle fractures and the clinical relevance and treatment of syndesmosis injury

1431. Lavander, Päivi (2017) Nimikesuojattujen ja laillistettujen ammattihenkilöiden työnjako yliopistosairaalan muuttuvassa toimintaympäristössä

1432. Vihanninjoki, Kyösti (2017) The Heidelberg Retina Tomograph in the diagnosis of glaucoma

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1435. Ramsay, Hugh (2017) Predictors of psychosis risk and neurocognitive deficits

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1437. Filatova, Svetlana (2017) Incidence of schizophrenia and associations of schizophrenia and schizotypy with early motor developmental milestones

1438. Käräjämäki, Aki (2017) Non-alcoholic fatty liver disease (NAFLD) : perspectives to etiology, complications and lipid metabolism

1439. Mikkola, Reija (2017) Determinants and clinical implications of bleeding related to coronary artery bypass surgery

1440. Hagnäs, Magnus (2018) The association of cardiorespiratory fitness, physical activity and ischemic ECG findings with coronary heart disease-related deaths among men

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ISBN 978-952-62-1764-2 (Paperback)
ISBN 978-952-62-1765-9 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)