LINKÖPING UNIVERSITY MEDICAL DISSERTATIONS NO. 1382

**IDENTIFICATION OF TUMOR CELL- AND STROMA DERIVED
BIOMARKERS OF TREATMENT RESPONSE IN HEAD AND NECK
CANCER**

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To my family, both two- and four-legged

“Nothing is impossible, the impossible only takes longer.”

Winston Churchill

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) poses a major health problem in the world with approximately 600 000 new cases yearly. Treatment resistance is a major problem within this patient group and despite advances in treatment strategies the overall survival rate has unfortunately not increased.

One of the major components of the tumor microenvironment is the cancer associated fibroblasts (CAFs) which can modulate the treatment sensitivity, tumor growth, and the invasive potential of tumor cells.

The aim of this thesis was to identify predictive markers for treatment response in HNSCC and to study the crosstalk between tumor cells and CAFs that may underlie treatment resistance.

In paper I, we identified gene expression differences between one cisplatin sensitive cell line and two cisplatin resistant cell lines, by microarray analysis, and found that a high expression of matrix metalloproteinase (MMP) -7 was associated with resistance to cisplatin. In paper II, the epidermal growth factor (EGF) receptor ligands EGF, amphiregulin, and epiregulin were evaluated regarding their potential use as predictive biomarkers for cetuximab treatment response in tongue cancer cell lines and it was shown that EGF may serve as a marker for poor cetuximab response. In paper III and IV, we investigated the influence of CAFs on the proliferation, migration, gene expression, and cetuximab response of tumor cells. It was found that CAFs induced resistance to cetuximab in a MMP-dependent manner. In addition, a microarray analysis,

comparing tumor cells co-cultured with CAFs and tumor cells cultured alone, revealed that CAFs induced multiple gene expression changes in tumor cells some of which are related to epithelial to mesenchymal transition. Some of these changes were found to be dependent on cell-cell contact.

Taken together, we here suggest MMP-7 and EGF to be predictive markers of cisplatin and cetuximab response, respectively. We also show that CAFs protect HNSCC cells from cetuximab treatment; however, the factor responsible for the protective effect is yet to be discovered.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer i huvud- halsregionen är den sjätte vanligaste cancertypen i världen och omfattar tumörer i tunga, kind, tandkött, halsmandlar, stämband, struphuvud och bihålor. Två av de största riskfaktorerna för denna cancertyp är rökning och högt alkoholintag. I kombination med varandra ökar risken för huvud- halscancer väsentligt eftersom alkohol har visats förstärka de cancerframkallande effekterna med rökning. Användandet av viss rökfri tobak, såsom t.ex. tuggtobak, ökar också risken för huvud- halscancer väsentligt, dock har inte någon ökad risk vid användning av det svenska snuset påvisats. En annan riskfaktor som har blivit uppmärksammas de senaste åren är infektion av humant papillomvirus (HPV), vilket är samma virus som orsakar cancer i livmoderhalsen. Förekomsten av huvud-halstumörer orsakade av HPV har ökat kraftigt på senaste tiden. Förändrade sexualvanor tros vara orsaken till denna ökning.

Den vanligaste behandlingen för huvud- halscancer i Sverige är strålning alternativt strålning i kombination med operation. På senare år har även cellgifter, såsom cisplatin, börjat användas i kombination med strålning och nya målinriktade behandlingar, t.ex. Erbitux, har utvecklats. Syftet med dessa nya läkemedel är att de ska påverka och/eller stänga av specifika egenskaper hos cancercellen som t.ex. cellens förmåga att dela sig och växa.

I en tumör finns inte enbart cancerceller utan även andra typer av celler som påverkar tumörens svar på behandling. En av dessa celltyper är bindvävscellerna, vilka normalt är involverade i bl.a. sårhäkning och bildar ärr. I

cancer är dock bindvävscellerna ständigt aktiva och därför brukar cancer liknas vid ett sår som aldrig läker.

Eftersom tumörerna i denna cancertyp involverar huvud- halsregionen påverkas patienternas tal- och sväljfunktion samt andning avsevärt under behandlingen, vilket gör den till en av de mest plågsamma cancertyperna. Trots att behandlingarna har förbättrats det senaste decenniet är det många patienter som inte svarar på behandlingen och därmed endast upplever de svåra biverkningarna. Därför är det väldigt viktigt att kunna urskilja de patienter som har goda förutsättningar att svara bra på en viss behandling, men även de som med största sannolikhet inte kommer att gynnas av behandlingen. Detta för att kunna välja bästa behandling för varje enskild individ samt undvika onödigt lidande hos patienterna.

Målet med denna avhandling var att för behandlingssvar hitta prediktiva markörer, d.v.s. om cancercellernas olika innehåll (t.ex. proteiner eller andra molekyler) kan förutspå vilka patienter som kommer att svara på en särskild behandling respektive inte svara på behandling. I denna avhandling visar vi att en hög mängd av ett protein som kallas matrix metalloproteinas-7 (MMP-7) är kopplat till resistens mot cisplatin, det krävs dock fler studier för att bevisa att MMP-7 kan användas som en prediktiv markör för cisplatinresistens. Som tidigare nämnt påverkar de omgivande bindvävscellerna tumörens svar på behandling och vi visar i avhandlingen att dessa bindvävsceller har god förmåga att skydda cancercellerna från den målinriktade behandlingen Erbitux.

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- I **Anna Ansell**, Fredrik Jerhammar, Rebecca Ceder, Roland Grafström, Reidar Grénman, and Karin Roberg
Matrix metalloproteinase-7 and -13 expression associate to cisplatin resistance in head and neck cancer cell lines
Oral Oncol. 2009 Oct;45(10):866-71.
- II **Anna Ansell**, Adam Jedlinski, Ann-Charlotte Johansson, and Karin Roberg
Epidermal growth factor is a biomarker for poor cetuximab response in tongue cancer cells
Submitted to Head & Neck.
- III Ann-Charlotte Johansson, **Anna Ansell**, Fredrik Jerhammar, Maja Bradic-Lindh, Eva Munck-Wikland, Reidar Grénman, Arne Östman, and Karin Roberg
Cancer-associated fibroblasts induce matrix metalloproteinase-mediated cetuximab resistance in head and neck squamous cell carcinoma cells
Mol Cancer Res. 2012 Sep;10(9):1158-68.
- IV **Anna Ansell**, Matti Kankainen, Jan-Ingvar Jönsson, Outi Monni, Karin Roberg, and Ann-Charlotte Johansson
Molecular cross-talk between head and neck squamous cell carcinoma cells and cancer-associated fibroblasts
Manuscript.

Other publications not included in this thesis

1. **Anna Ansell**, Lovisa Farnebo, Reidar Grénman, Karin Roberg, and Lena K Thunell
Polymorphisms of FGFR4 in cancer development and sensitivity to cisplatin and radiation in head and neck cancer
Oral Oncol. 2009 Jan;45(1):23-9.
2. Lovisa Farnebo, Adam Jedlinski, **Anna Ansell**, Linda Vainikka, Lena K Thunell, Reidar Grénman, Ann-Charlotte Johansson, and Karin Roberg
Proteins and single nucleotide polymorphisms involved in apoptosis, growth control, and DNA repair predicts cisplatin sensitivity in head and neck cancer cell lines
Int J Mol Med. 2009: Oct;24(4):549-56.
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Acidic preparations of lysed platelets up-regulate proliferative pathways in osteoblast-like cells as demonstrated by genome-wide microarray analysis
Platelets. 2011;22(6):452-60.
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EGFR status and EGFR ligand expression influence the treatment response of head and neck cancer cell lines
J Oral Pathol Med. 2012: Jan;42(1):26-36.
5. Lovisa Farnebo, Katarina Tiefenböck, **Anna Ansell**, Lena K Thunell, Stina Garvin, and Karin Roberg
Strong expression of survivin is associated with response to radiotherapy and improved overall survival in head and neck squamous cell carcinoma patients
Int J Cancer. 2013 Oct 15;133(8):1994-2003.

ABBREVIATIONS

AR	Amphiregulin
CAF	Cancer associated fibroblast
CM	Conditioned medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
EPR	Epiregulin
FACS	Fluorescence-activated cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FDA	Food and drug administration
GOTM	Gene ontology tree machine
HB-EGF	Heparin-binding EGF-like growth factor
HGF	Hepatocyte growth factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
ICS	Intrinsic cisplatin sensitivity
IPA	Ingenuity pathway analysis
MACS	Magnetic activated cell sorting
MMP	Matrix metalloproteinase

MPI	Matrix metalloproteinase inhibitor
mRNA	Messenger RNA
MT-MMP	Membrane-type matrix metalloproteinase
NNK	Nicotine-derived nitrosamine ketone
NNN	N'-nitrosornicotine
NSAID	Non-steroidal anti-inflammatory drug
NSCLC	Non-small cell lung cancer
PAH	Polycyclic aromatic hydrocarbons
PFA	Paraformaldehyde
qPCR	Quantitative real-time PCR
Rh	Recombinant human
SCC	Squamous cell carcinoma
SFM	Serum free medium
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
ssRNA	Single-stranded RNA
TIMP	Tissue inhibitor of metalloproteinase
TNM	Tumor, nodes, metastases
TSNA	Tobacco-specific nitrosamine

INTRODUCTION

Head and neck cancer

Head and neck cancer is a broad term referring to malignant neoplasms of the upper aerodigestive tract.^{1,2} These tumors are further subdivided according to the area in which they arise (Figure 1).

Figure 1. A schematic picture of the different sites in the upper aerodigestive tract where head and neck tumors can arise; the **oral cavity** (including the lips, tongue, gingiva, buccal mucosa, floor of mouth, and the hard palate), **pharynx** (including the nasopharynx, oropharynx, and hypopharynx), **larynx**, **paranasal sinuses**, **nasal cavity**, and **salivary glands**.

Approximately 90% of these tumors originate from the epithelial cell layer and are thus, so-called squamous cell carcinomas (SCC), which are frequently aggressive in their biological behavior.² Two-thirds of the patients have advanced disease with lymph node metastasis and poor prognosis. Patients with early-stage tumors have a better prognosis but unfortunately cancer in the head and neck region is usually diagnosed at a late stage.

SCC is the only patho-anatomic diagnosis studied in this thesis.

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer worldwide for males and the eight for women with approximately 600 000 new cases and 350 000 deaths every year.³ Cancer in the nasopharynx is usually separated from other HNSCC since it has a specific aetiology to Epstein-Barr virus⁴ and genetic factors affecting the immune response.⁵ However, if adding these tumors to the HNSCC group it would push head and neck cancer higher up the scale of most common cancers worldwide.⁶

The incidence of the different types of HNSCC displays a diverse geographical pattern worldwide.⁶ Due to differences in risk factors, diet, and health care among countries, two-thirds of HNSCC cases are found in developing countries. High-risk regions for cancers in the oral cavity includes Melanesia, south central Asia, western and southern Europe, Australia, and southern Africa. The enormously high risk of oral cancer in Melanesia has not been researched but data from nearby island Papua New Guinea suggest betel chewing as the major risk factor. The same risk factor causes the high prevalence of nasopharyngeal cancer in Micronesia and southeastern Asia. South central and southeastern Asia, South America, the Caribbean, and Europe, excluding the northern regions, are high risk regions for larynx cancer. These regions have high smoking prevalence explaining the high incidence of laryngeal cancer.

There are not only ethnical differences among HNSCC cases but also differences between genders. Males are overall at higher risk to develop cancer in the head and neck region⁶ probably because of their more excessive use of alcohol and tobacco than females. However, studies have shown that women who are smoking have a larger increased risk of developing HNSCC than males who are smoking.⁷ The mechanisms underlying these differences are not yet fully understood but hormonal risk factors could be involved. Indeed, estrogen deficiency has been shown to increase the risk for oral cancer among women.^{8,9}

The typical HNSCC patient is a 65 year old male with low socioeconomic status and heavy alcohol and smoking habits. However, an alarming trend has been noticed the past years with an increasing number of individuals younger than 45 years of age developing HNSCC.^{10,11} It is not clear why this increased incidence among young adults occur. Some case reports have though come to the conclusion that the risk factors present in older patients were similar to the ones in younger patients^{11,12} and that early exposure of the oral epithelium to risk factors such as tobacco and alcohol may shorten the latency period in carcinogenesis.¹³ As noticed, the highest risk for developing oral cancer among young adults was onset of smoking and excessive drinking under the age of 16 years.¹² In other parts of the world where the prevalence for head and neck cancers are high many individuals who are less than 40 years old develop cancer owing to cultural practices with early onset of use of different forms of tobacco. On the other hand, there is a distinct patient group, encompassing primarily younger females, which have little or no exposure to any major risk factors.¹⁴⁻¹⁶ This group calls for further studies but genetic susceptibility¹⁷ and younger mean age at menopause, leading to estrogen deficiency, have been suggested as risk factors.^{8,9} In recent years, the human papillomavirus (HPV) type 16 has been

associated with cancer in the tonsils and base of tongue in a subset of younger patients with no other reported risk factors.^{18,19}

Risk factors

One of the most important risk factors for HNSCC is tobacco²⁰ which can be consumed in many different forms; cigarettes, cigars, beedi, oral snuff, or in moist pouches (used in northern Scandinavia).²¹

Smoking tobacco

Smoking tobacco is a worldwide problem with nearly 1.3 billion users.²² Cigarette smoking is strongly associated with oral, larynx, and pharynx cancer.²³ To date, more than 60 carcinogenic products have been found in cigarettes. Tobacco-specific nitrosamines (TSNAs), which include nicotine-derived nitrosamine ketone (NNK) and N'-nitrosonornicotine (NNN), and polycyclic aromatic hydrocarbons (PAH) are the most carcinogenic products found in cigarettes and also the most causative agents for oral, larynx, and pharynx cancer. NNK, NNN, and PAH contribute to the development of cancer by formation of DNA adducts.²⁴ In a normal state, the cellular repair system removes the DNA adducts, but if this system is malfunctioning mutations may arise e.g., PAH has been associated with mutations in the tumor suppressor gene p53 leading to larynx cancer. Oxidative stress can be caused by cigarette smoke due to the content of free radicals such as nitric oxide. Free radicals are known to damage proteins, lipids, and DNA.^{25,26}

Smokeless tobacco

Smokeless tobacco is a significant and growing problem worldwide and there are many different types of products. In contrast to smoking tobacco, smokeless tobacco contains around 16 carcinogenic products and the levels of PAH are

quite low.²⁷ However, the NNKs and NNNs are hundreds to thousands times higher than in cigarettes and are the most important agents in the development of oral cancer in smokeless tobacco users. As recently noticed, NNK together with nicotine binds to different cell surface receptors such as the nicotinic-acetylcholine receptor, thus activating the AKT-pathway, leading to decreased apoptosis and increased cell proliferation. Conversely, the moist pouches used in northern Scandinavia have a lower content of TSNA and it has been suggested as a nicotine replacement in smokers who are having difficulties in smoking cessation.^{28,29} This is however, highly controversial since no safe tobacco exists to date.

Since tobacco is the most important cause of cancer and cancer-related deaths, it has been used as a model for understanding the mechanisms of cancer development.

Betel quid chewing

Betel quid and areca nut chewing are practiced by 600 million people worldwide.³⁰ It is widespread in many parts of Asia and among Asian-immigrants in other parts of the world. A betel quid is commonly composed of betel leaf from the Piper betel vine, areca nut from the Areca catechu tree, slaked lime (calcium hydroxide), and spices according to local preferences. The betel quid may also include tobacco. The areca nut contains at least four chemical alkaloids whose nitrosamine derivatives have been found to be carcinogenic.³¹ Approximately 40% of all areca nut samples are contaminated with the fungus *Aspergillus flavus*³² which contributes to poor oral hygiene. Subjects with poor oral hygiene have an increased formation of endogenous nitrosamines³¹ and this enhances the amount of nitrosamines upon betel quid chewing.³³ As seen in tobacco smoking, betel quid chewing can also cause

oxidative stress.²⁵ In addition, slaked lime causes erosions of the oral mucosa and this facilitates the infiltration of betel quid carcinogens.³⁴

Alcohol

One of the other major risk factors for oral cancer is alcohol consumption.^{35–37} Alcoholic beverages are usually composed of ethanol and water and although ethanol itself is not carcinogenic, acetaldehyde, a metabolite of ethanol, has mutagenic and carcinogenic effects.³⁸ When acetaldehyde is produced from ethanol by mucosal alcohol dehydrogenase in the oral epithelium it interferes with DNA synthesis and repair which may lead to mutations.^{35,39} Alcohol is also known to greatly enhance the risk for oral cancer when it is used together with tobacco.^{40–42} Alcohol may render the tissue more sensitive to tobacco carcinogens either by increasing their solubility³⁹ or by increasing the permeability of the oral mucosa.⁴³ Furthermore, smokers were found to have increased amounts of gram-positive bacteria, capable of producing acetaldehyde from ethanol, in their oral epithelium.^{44,45} This may also contribute to the synergistic effect between alcohol and tobacco.

Whether mouthwashes containing alcohol might be a risk factor for oral cancer is an interesting question. However, so far epidemiological studies have not found a link between the use of alcohol-containing mouthwashes and oral cancer.^{46,47}

Diet and nutrition

Dietary factors are the cause of 30% of all cancers in the world⁴⁸ and it is also one of the most important risk factors for HNSCC of all sites^{49–51} together with tobacco and alcohol. High intake of fresh fruits and especially citrus fruits, vegetables, fish, and vegetable oils has been associated with a low risk of developing HNSCC.⁴⁹ All of these are rich in vitamins A, C, and E, beta-

carotene, and selenium. These micronutrients have antioxidant effects and reduce free radical reactions.^{52,53} However, high intake of micronutrients does not decrease the risk of developing HNSCC due to smoking and alcohol consumption.⁵⁴ There are many differences in diets between ethnical groups due to cultural food habits. In addition, there are also large cultural differences when it comes to the use of risk factors. For example, there are more blacks than whites suffering from HNSCC in the USA. Studies revealed that blacks tended to use alcohol and tobacco to a higher extent and that the intake of fresh fruits were much lower among the blacks.^{41,42} Furthermore, it has been shown that smoking and alcohol drinking reduce the levels of beta-carotene and vitamin C in serum.⁵⁵⁻⁵⁷ Speculatively, heavy drinkers may also have nutritional deficiencies due to decreased micronutrient intake, or weakened absorption. During betel quid chewing lower levels of vitamins A and C, and beta-carotene have also been noticed.⁵⁸ However, if the betel quid contained betel leaves, betel chewers were found to have high levels of beta-carotene. This may be explained by the fact that betel leaves contain several phenols which have antioxidant effects and might to some extent be protective.

Genetic predisposition

Since not all smokers or heavy drinkers develop HNSCC, exposure to carcinogens cannot alone fully account for the development of cancer and it has been suggested that some individuals may have an inherited genetic susceptibility to HNSCC.¹⁷

Although inherited HNSCC is not very common there is evidence for inherited genetic predispositions causing HNSCC. These inherited genetic alterations can either be conditions with a predisposition to cancer such as Fanconis anaemia, dyskeratosis congenita, and Cowden syndrome^{59,60} or multiple single nucleotide polymorphisms (SNPs) which cause DNA damage, activation of oncogenes,

inactivation of tumor suppressor genes, loss of function in the DNA repair system, or impaired carcinogen-metabolizing enzyme systems such as the cytochrome P450 and the glutathione S-transferase mu 1.^{61–63} All these genetic alterations lead to genomic instability, the most constant characteristic of cancer.

Human papillomavirus

As previously mentioned, an increase of younger patients developing HNSCC has emerged the past few decades and an increase of oropharyngeal cancers, i.e., cancer of the tonsils and base of tongue, have been noticed within this younger patient group and especially in men.^{64,65} These patients commonly report no history of tobacco or alcohol use so other risk factors may have contributed to the observed trend. Indeed, this malignancy has recently been reported to be associated with HPV and mainly HPV type 16.¹⁹ A large multi-center study has shown that more than 25% of all HNSCC contain HPV genomic DNA⁶⁶ and that 72% of all oropharyngeal cancers are HPV-positive.¹⁹ However, here in Sweden the incidence of HPV-positive oropharyngeal cancers is much greater.^{67,68} Researchers at Karolinska Institute have shown that the proportion of HPV-positive tonsillar cancers has increased from 68% during the years 2000-2002 to 93% during 2006-2007.⁶⁷ This increased incidence of HPV-positive tumors may be explained by changes in sexual norms such as having more oral sex partners or having oral sex at an earlier age. In addition, the prevalence of HPV is higher in cervical cancers than in penile cancers, making the speculation that performing oral sex on women boosts the risk for HPV infections in men. Thus, men have a higher rate of HPV associated oropharyngeal cancers. A recent study has also shown that individuals with reported poor oral health had a 56% higher prevalence of HPV-infections.⁶⁹ A poor oral hygiene may include ulcers or inflammations in the gingiva and this might facilitate the entry of HPV since this virus usually requires wounds to establish infections.

High-risk HPV such as HPV type 16 mediate their carcinogenic effect through the action of the two viral oncogenes E6 and E7.⁷⁰ E6 binds and forms a complex with p53 which results in p53 degradation⁷¹ while E7 destabilizes the Rb tumor suppressor protein.⁷² Both of these events result in defects in apoptosis, DNA repair, and cell cycle control and this leads down the pathway towards malignancy.

Two vaccines for prevention of HPV-related diseases have been developed: Cervarix ® (Glaxo Smith Kline), which is a vaccine against HPV type 16 and HPV type 18, and Gardasil® (Merck & Co) which is also protective against HPV type 16 and HPV type 18. In the future it will be very interesting to see if these new vaccines will protect not only against cervical cancer but also against HPV-positive HNSCC.

Treatments for head and neck cancer

Despite advances in surgical and oncological treatments that enhance quality of life the overall survival rate has unfortunately not increased over the past decades and clinical drug resistance remains a major problem in HNSCC.⁷³ The difficulties in HNSCC treatment might be explained by the high heterogeneity within the group of HNSCC tumors. Tumors of the oral cavity (including lips, tongue, gingiva, buccal mucosa, floor of mouth and the hard palate), pharynx, larynx, and paranasal sinuses all belong to the same group of cancers despite their differences in location, cause and/or function. These differences may also cause the complexity in finding markers predictive for treatment responses. To date, factors that influence the choice of treatment are the characteristics of the primary tumor which include, TNM (tumor, nodes, metastases) -stage, location, proximity to bone, and histological grading of the tumor.⁷⁴ In addition, factors related to the patient including age, general medical condition (which might

affect the tolerance of treatment), lifestyle (smoking, drinking and other socioeconomic difficulties), and acceptance by the patient are also taken into consideration when determining the initial treatment. Expertise within the team of physicians is also an important factor in making treatment decisions since managing HNSCC is a multidisciplinary team effort. Knowledge in surgery, radiotherapy, chemotherapy, rehabilitation, dental-, and psycho-social support all play an important role in the treatment selection.

The curative management for locally advanced HNSCC in stages III or IV is more difficult than for early stage HNSCC (stage I or II). Stage III or IV disease is associated with a decrease in loco-regional control, an increase in distant metastasis, and a shorter disease-free survival, and generally demands more intense treatment when compared to early stage disease.

Surgery

Surgery is considered to be the standard treatment for HNSCC patients with early stage I or II cancer in the oral cavity.¹ However, the anatomical location of head and neck tumors makes surgery a challenge since major functions such as swallowing and speech are affected by the treatment.⁷⁵ In the past decade organ preservation methods such as microvascular free-flaps, which aims to maintain these functions, have increased.^{2,76}

Radiation

Radiation is the standard treatment for HNSCC patients with early stage I or II oropharyngeal and hypopharyngeal cancers.¹ For managing locally advanced HNSCC (stage III or IV) the primary mode of treatment is surgery in combination with pre- or post-operative radiation. Since the overall survival after radiation is less than 25%^{77,78} a number of efforts have been made to improve these disappointing results. Two altered fractionation radiotherapies,

hyperfractionation and accelerated fractionation, have been introduced, however, the results have not been ground-breaking.^{79–81} Many factors such as smoking habits during treatment, hemoglobin levels, hypoxia levels, and tumor location have been implicated to affect the radiation treatment outcome.⁸²

The exposure of cells to ionizing radiation leads to DNA damage through the formation of reactive oxygen species that chemically react with DNA.⁸³ The most severe damage is the double-strand break, which results in cell cycle arrest, apoptosis, gene inactivation, reproductive failure, or terminal senescence.

Chemotherapy

Chemotherapies such as cisplatin, carboplatin, docetaxel, gemcitabine, and fluorouracil have evolved from being used in palliative care to be used also as curative components in the treatment of locally advanced HNSCC.⁸⁴ However, cisplatin is the most common and effective chemotherapeutic drug used in the treatment of locally advanced HNSCC. It was discovered by accident in 1965 when Barnett Rosenberg applied electromagnetic radiation using platinum electrodes to *Escherichia coli* and saw that the bacteria filaments grew up to 300 times their normal length.^{85,86} The cell growth was continued during the radiation through the platinum electrodes but the cell division was inhibited. What they realized after this experiment was that they had rediscovered a platinum coordination complex, known as Peyrone's chloride, that was originally synthesized and described in 1845.⁸⁷ The effect on cell division was later verified in a sarcoma mouse model where cisplatin caused marked tumor regression.⁸⁸

Cisplatin exerts its anti-cancer effects by binding covalently to DNA and forming DNA adducts which results in activation of DNA repair, cell-cycle arrest, and apoptosis.⁸⁶ Clinical resistance to cisplatin is a significant problem

for patients with locally advanced HNSCC and only 20-30% of the patients respond to this treatment.⁸⁹ Numerous cellular mechanisms have been implicated in resistance to cisplatin and these include decrease in cellular drug accumulation, increased DNA repair, increased levels of intracellular thiols (e.g., glutathione or metallothionein) that binds to cisplatin and causes deactivation, and increased expression, mutations, or deregulation of anti-apoptotic genes.⁹⁰⁻⁹³

Cisplatin has very severe side-effects including nephrotoxicity, ototoxicity, neutropenia as well as nausea and vomiting,⁹⁴ which all cause a lot of pain and distress within the patient. Speculatively, it is not clear whether cisplatin would even be approved if it was to be presented to regulatory authorities today.

Chemoradiotherapy

As previously mentioned, locally advanced HNSCC (stage III or IV) have a more aggressive behavior and require more intense treatments in order to achieve loco-regional control. The introduction of concurrent administration of chemotherapy and radiotherapy has been a major advancement for late stage disease.¹ Although several phase III clinical trials have shown that chemoradiotherapy improve loco-regional control, the absolute survival benefits noticed in these studies are relatively small and can in general be explained by the improved loco-regional control.⁹⁵⁻¹⁰¹ Meta-analyses of clinical trials have showed that chemoradiotherapy compared to radiotherapy alone gave a slight increase in absolute survival benefit of 4-8% at 5 years however; this was related to increased toxicity including mucositis, dermatitis, and myelosuppression.¹⁰²⁻¹⁰⁴ Nevertheless, these toxic effects are overshadowed by the paramount importance to cure the patients and chemoradiotherapy is nowadays a standard treatment for locally advanced HNSCC.¹⁰⁵

Targeted therapy

Targeted therapies are a promising field in cancer managements and they are usually targeting growth factor receptors and their downstream signaling. One such receptor is the epidermal growth factor receptor (EGFR) which is overexpressed in more than 90% of all HNSCC.¹⁰⁶ Moreover, EGFR expression is associated with poorer survival and loco-regional failure. Due to the relationship between the overexpression of EGFR and the aggressive behavior of locally advanced HNSCC, therapies aiming to prevent EGFR signaling, including monoclonal antibodies and small molecule tyrosine kinase inhibitors have been developed. The monoclonal antibody cetuximab (Erbix[®], Merck KGaA) was in 2006 approved by the Food and Drug Administration (FDA) to be used in combination with radiotherapy as a first-line treatment for patients with loco-regionally advanced HNSCC.^{107,108} Cetuximab has also been approved for recurrent or metastatic HNSCC both as first-line treatment in combination with platinum-based chemotherapy,¹⁰⁹⁻¹¹² and as second-line treatment in platinum-refractory patients.¹¹³⁻¹¹⁵ Although cetuximab has shown promising results in HNSCC clinical trials the response rate is generally not higher than 20%.^{107,110,116,117} KRAS mutations, which are well-established markers of cetuximab resistance in colorectal cancers,¹¹⁸ are rarely present in HNSCC tumors;¹¹⁹ therefore, this marker has not proven useful for the selection of HNSCC patients to receive cetuximab treatment.

Cetuximab is associated with skin toxicity and the typical side-effects include rashes and changes to the hair and nails.¹⁰⁶ Following administration, cetuximab binds to the extracellular domain of EGFR and prevents ligand binding.¹²⁰⁻¹²² In addition, it may also stimulate the internalization of EGFR, which leads to the downregulation of its cell surface expression, and may also trigger antibody-dependent, cell-mediated cytotoxicity.¹²³

Since the side-effects during cetuximab treatment are not that severe it is used in combination with radiation for locally advanced HNSCC in patient who are not good candidates for cisplatin.

Quality of life in patients with HNSCC

Of all human cancers, HNSCC is the most devastating one causing a great deal of pain and suffering for the patients. As previously mentioned, patients with HNSCC and especially those with late stage disease have a high risk of treatment failure and if cured, they are facing disfigurement, loss of speech, and impairment of the basic survival functions of breathing and swallowing. This results in serious medical and psychosocial consequences and many patients may be at risk for severe depression or even worse, suicide. It is very important to capture the patient's point of view following HNSCC disease and treatment since that may differ from the physicians' opinion. In recent years, quality of life data has been used as a tool for measuring the patient's perspective.¹²⁴

Due to the anatomical location of HNSCC tumors the patient's smell, taste, speech, and ability to swallow may be disrupted before, during, and after treatment. Xerostomia, or dry mouth, is a major problem after radiotherapy and it has been shown that patients suffering from xerostomia also have a greater impairment in swallowing and speech performances. Pain is a specific concern within HNSCC patients and may arise as a result of many factors such as tumor ulcerations, tumor pressure effects, nerve infiltration, treatment-related pain due to radiotherapy and chemotherapy, postoperative wounds, shoulder dysfunction, or osteoradionecrosis. It is heartbreaking that many patients do not respond to the different treatments since these are responsible for the many sufferings mentioned above and this is why the search for markers that will predict treatment outcome is so important.

Biomarkers in cancer

A biomarker can either have a prognostic or a predictive value, or both. A prognostic marker foretells the risk of recurrence or survival in the absence of treatment or during the use of a standard therapy whereas a predictive marker guides the treatment decision since it predicts the outcome of specific treatments. An ideal biomarker would have both a prognostic and a predictive value and would also serve as a therapeutic target.

Numerous proteins and genetic markers (e.g., mutations, SNPs, and gene copy number) involving cell cycle control, apoptosis, and growth regulation have been proposed to refine clinical outcome and predict treatment outcomes in several cancer types over the past decade. Measurement of hypoxia and the detection of viral DNA have also been proposed as potential biomarkers. However, very few of these markers are in routine clinical use and it is unlikely that one single marker could truthfully predict response to treatment or have a prognostic value. How tumor cells progress and respond to treatments are very complex and involves many different deregulated pathways. Hence numerous markers have to be combined in order to predict survival and treatment outcome in cancer patients.

To date, no predictive marker or panel of markers has been validated to be used in the clinic for selection of treatment options for patients with HNSCC. In contrast, for colorectal cancers KRAS mutation status is predictive for the selection of patients receiving cetuximab¹¹⁸ but this is not an option for HNSCC since these mutations are very rare in this disease.¹¹⁹ In addition, the estrogen receptor and progesterone receptor are used as predictive markers for selecting breast cancer patients likely to respond to hormone therapy.¹²⁵ Another predictive marker used in metastatic breast cancer is the gene amplification or

overexpression of human epidermal growth factor receptor 2 (HER2) which identifies individuals who may benefit from trastuzumab.^{126–128} HER2 belongs to the same subclass of receptor tyrosine kinases as EGFR¹²⁹ but gene amplification or overexpression of EGFR are not predictive for cetuximab treatment outcome in HNSCC.^{130,131} On the contrary, a high EGFR expression has been shown to predict survival benefits in patients with advanced non-small cell lung cancer (NSCLC) who receive cetuximab in combination with first-line chemotherapy.¹³² Nevertheless, in HNSCC patients, a high EGFR expression has been associated with poor prognosis and decreased overall survival and is therefore used as a prognostic marker in HNSCC.^{133,134} One hypothesis as to why EGFR expression is not predictive of cetuximab treatment outcome is that clinical doses of cetuximab may not achieve receptor saturation within tumors with high EGFR expression. Furthermore, in a study where EGFR expression was determined by immunohistochemical analysis it was shown that tumors with less than 80% cellular EGFR positivity had a better response to cetuximab.¹¹² Interestingly, to date, the strongest biomarker for cetuximab treatment response in HNSCC is the clinical observation of skin rashes and not a laboratory-based assay.¹¹² Patients with skin rashes have a much better response to cetuximab treatment than patients who do not develop skin toxicities. However, this biomarker is only predictive during treatment and is therefore impossible to use as a predictive marker before treatment.

HPV type 16 is now also recognized as a prognostic marker for survival outcome in HNSCC; patients with HPV-positive tumors have a better prognosis than patients with HPV-negative tumors.¹³⁵ This is likely due to the fact that the biology of HPV-associated tumors is different from that of tobacco-associated HNSCC. For example, tobacco-related HNSCC is associated with loss of p16 and overexpression of cyclin D1^{136,137} whereas, in HPV-related HNSCC,

overexpression of p16 and low levels of cyclin D1 is documented.¹³⁸ Inactivation or deletion of p16 and overexpression of cyclin D1 have been associated with poor prognosis, reduced survival, and impaired response to treatment.^{136,139} What is more, patients with HPV-positive tumors seem to be more responsive to both radiotherapy and chemotherapy¹⁴⁰ however, the use of HPV type 16 as a predictive marker for treatment outcome in HNSCC has not yet been proven. The overall better prognosis regardless of the treatment regimen for HPV-positive HNSCC compared with HPV-negative HNSCC seems to be related to the immune system rather than the tumor cells' intrinsic sensitivity to the treatments.^{141,142}

The lack of predictive markers in HNSCC is worrying and a great challenge for researcher within the field. HNSCC is a very heterogeneous disease which hampers accurate prognostication, treatment planning, and, from a biomarkers point of view, the identification of causative cancer genes. The increased availability of high-throughput screening methods and the combinations of other molecular analyses will hopefully lead to the discovery of markers associated with prognosis and treatment outcome in HNSCC.

Epidermal growth factor receptor in cancer

EGFR is overexpressed in approximately 30% of all human epithelial tumors¹⁴³, including HNSCC, of which nearly all tumors exhibit EGFR overexpression.¹⁴⁴ EGFR is a transmembrane tyrosine kinase cell surface receptor that belongs to the ErbB family, which consists of four closely related receptors: EGFR (ErbB1/HER1), ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4).^{129,145} Upon ligand binding, these receptors (except for HER2 which has no known ligand¹⁴⁶) form either homo- or heterodimers, which results in activation of the intracellular tyrosine kinase domain.¹⁴⁷ The EGFR/HER2 heterodimer is more

highly expressed on the cell surface than EGFR/EGFR homodimers and it is also more stable, leading to a prolonged activation period.^{148,149} The subsequent triggering of several downstream signaling pathways, including MAPK, PI3K/Akt, JAK/Src/STAT, and PLC γ , leads to the transcription of genes associated with proliferation, migration, and survival.^{150,151}

Figure 2. Upon ligand binding the epidermal growth factor receptor (EGFR) dimerizes, which leads to the autophosphorylation and activation of the intracellular tyrosine kinase domain. This leads to triggering of multiple downstream signaling pathways including MAPK, PI3K/Akt, JAK/Src/STAT, and PLC γ .

There are seven known EGFR ligands: epidermal growth factor (EGF), amphiregulin (AR), epiregulin (EPR), betacellulin, epigen, transforming growth

factor- α , and heparin-binding EGF-like growth factor (HB-EGF).¹⁵² These ligands are secreted by tumor cells and participate in auto- and paracrine stimulation.¹⁵³

Why some tumors do not respond to treatment with the monoclonal EGFR antibody cetuximab is not completely understood but numerous underlying mechanisms have been proposed during the years. The EGFR variant III, which is a truncated form of EGFR that lacks the ligand binding domain and is constitutively active, has been shown to contribute to both cetuximab and cisplatin resistance.¹⁵⁴ The formation of EGFR heterodimers and/or crosstalk with HER2, HER3, cMET, and insulin-like growth factor 1 receptor might also be responsible for resistance to cetuximab.¹⁵⁵ Other mechanisms include upregulation of EGFR ligands^{156–163} and higher expression of nuclear EGFR than membrane EGFR.¹⁶⁴ Epithelial to mesenchymal transition (EMT) was first implicated in resistance to cetuximab in hepatocellular carcinoma and urothelial carcinoma^{165,166} and current evidence show that also HNSCC cells may utilize EMT in order to evade cetuximab treatment.^{167,168} This is likely due to lower EGFR expression (which leads to decreased EGFR-dependent survival signals) on mesenchymal-like cells.¹⁶⁷

Matrix metalloproteinases in cancer

The matrix metalloproteinase (MMP) family is a diverse group of approximately 23 different zinc-dependent endopeptidases which are synthesized as latent enzymes and are activated after release of their propeptide domains.^{169,170} The majority of MMPs are secreted proteins but an exception is the membrane-type metalloproteinases which are attached to the cell surface. The MMPs are further grouped into collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (MT-MMPs), and others.¹⁷⁰ Their local activity in tissues is

regulated by the expression of endogenous tissue inhibitors of metalloproteinases (TIMPs). Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified and TIMP-1 and TIMP-2 are commonly detected in HNSCC. Changes of TIMP levels during pathological conditions such as cancer are considered to be important since they directly affect the level of MMP activity.

MMPs have for a long time been considered as critical for tumor progression, invasion, and distant metastasis.¹⁷¹ At first, the understanding of MMP action in tumor progression and invasion was very simple; tumor cells produced and secreted MMPs in order to degrade the various components of the extracellular matrix (ECM) and thereby facilitate tumor cell spread to local and distant sites. However, our understanding has changed over the past years. It is now known that MMPs also have substrates unrelated to ECM components and that it is primarily the cleavage of these non-matrix substrates (e.g., chemokines, growth factors, growth factor receptors, integrins, and apoptotic mediators) that leads to tumor progression and invasion. Thus, MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer.^{172,173} Furthermore, it is also recognized that MMPs are synthesized not only by tumor cells but also by many other cell types within the tumor microenvironment such as endothelial cells, fibroblasts, and infiltrating immune cells.^{174,175}

There is no doubt that MMPs play an important role in cancer progression, invasion and metastasis and this has led to the clinical development of multiple MMP inhibitors (MPIs).¹⁷¹ Unfortunately, none of these MPIs showed positive results in phase II and III clinical trials. What is now known is that the development of MPIs in the 1990's had to overcome multiple unpredicted problems. First, at that time it was still unknown if specific MMPs played

important roles in different cancer types and the rapidity in which MPIs moved into clinical trials did not allow for expression analysis of the entire MMP-family in the specific cancer type used for clinical trials. For example, the MPI tanomastat, which targets MMP-2 and has very low activity toward MMP-11, should never have been tested in patients with non-small lung cancer since in this cancer type, high MMP-11 is associated with poor prognosis and MMP-2 is not detected. This particular trial was terminated early because tanomastat-treated patients showed poorer survival than placebo-treated patients. Another problem with the hasty progression of MPIs from phase I studies into phase II/III combination clinical trials was that the information regarding the benefit of efficacy from smaller studies was lost. Early phase I studies revealed that prolonged treatment with MPIs caused musculoskeletal pain and inflammations, thus clinical dosage of MPIs was determined due to these side effects rather than efficiency on tissue penetration or inhibition activity. It has been shown that inhibition of related metalloproteinases such as ADAMs and ADAMTSs are responsible for the side effects observed during administration of these broad spectrum MPIs.¹⁷⁶ Lastly, these clinical trials excluded patients with early stage cancer since the selection was based on the criteria for conventional chemotherapy. This is contradictory since MPIs seem to be more efficient in early stage cancer^{177,178} and that MMPs play an important role in early cancer development as well.^{172,173} MPIs are cytostatic drugs and should, therefore, not be compared with cytotoxic drugs.

MPIs were one of the first targeted therapies designed and tested in clinical trials but the outcome has been very disappointing. Many problems with these clinical trials have been brought to light but a wider understanding of the role of specific MMPs in tumors and the design of specific MPIs which do not have dose-limiting musculoskeletal side effects are wished for. One new possible

target for the area of MPIs could be the MT1-MMP which plays a critical role in cancer invasion. Interestingly, HNSCC is consistently related to MT1-MMP overexpression^{179–181} and was also one of the first human cancers associated with MT1-MMP expression.¹⁸²

The tumor microenvironment

Rather than being malignant cells growing in isolation, cancer is a complex tissue where many different cell types (e.g., fibroblasts, endothelial cells, pericytes, and inflammatory cells) and ECM interact in a multipart ecosystem, called the tumor microenvironment. Even though Paget hypothesized of the seed and the soil already in 1889,¹⁸³ it is only recently accepted that cancer progression is a product from crosstalk between different cell types within the tumor and its surrounding tumor stroma.^{184–186}

Figure 3. The tumor microenvironment consists of many different cells such as fibroblasts, endothelial cells, pericytes, and inflammatory cells, as well as extracellular matrix (ECM). All these cells have properties that, if deregulated, can contribute to cancer progression.

Already in pre-malignant dysplasia, fibroblasts, endothelial cells, pericytes, and immune cells are recruited to the stroma. There are many similarities between these tissue changes and the changes associated with wound repair, thus, cancer is commonly abridged as “a wound that never heals”.¹⁸⁷ The progression from dysplasia into invasive cancer involves disruption of the basement membrane barrier which enables direct contact between the tumor cells, stromal cells, and ECM. During this progression the normal stroma undergoes a transformation into a reactive tumor stroma, i.e., an abnormal stroma that supports tumor growth and metastasis.^{188–191}

The most crucial element of the microenvironment is the cancer associated fibroblasts (CAFs) which are similar to fibroblasts involved in wound healing. CAFs derive from different subsets of cells including local fibroblasts, bone marrow-derived progenitor cells, and trans-differentiating epithelial cells.¹⁹² The term CAF is yet relatively poorly defined, and based mainly on the expression of certain markers such as α -smooth-muscle actin, vimentin, collagen I, fibroblast specific protein-1 and platelet-derived growth factor receptor.¹⁹³ Since CAFs may derive from many different cells, the expression of markers within CAF-populations is heterogeneous and CAFs are also likely to display functional differences due to their origin.

CAFs modulate the tumor's fate by increasing tumor growth,^{188,194,195} EMT,^{184,186,196,197} the invasive potential,^{198,199} and metastasis^{196,200} by secretion of soluble factors or by modification of ECM components.²⁰¹ In addition, the tumor stroma has also been suggested to modulate the drug sensitivity of cancer cells. Already in 1990 Teicher proposed that something other than the tumor cells' properties must affect their ability to resist cytotoxic drugs.²⁰² In this study, mice bearing mammary tumors were over a 6-month period repeatedly treated with alkylating agents, resulting in resistance to the drug. However, this acquired drug resistance could only be seen *in vivo* and not in *in vitro* tumor cell culture, demonstrating that the surrounding tissue of the tumor, the tumor stroma, was influencing the tumor cells ability to resist cytotoxic drugs. Since then, numerous studies have shown that several different cell types within the tumor stroma, including CAFs, can influence the response to various anti-cancer treatments.^{203–211}

It is now clear that an abnormal stroma contribute to, or even is required for, tumor formation, progression, invasion, and resistance to anti-cancer treatments. One approach to slow or reverse this could be the design of targeted therapies

aiming to normalize components of the stromal environment. Stromal cells, such as CAFs, are good therapeutic targets since these cells are more genetically stable than tumor cells and thus, are more likely to maintain sensitivity to drugs.^{212,213} In fact, there already exists drugs approved by the FDA that targets the tumor stroma. Bevacizumab, i.e., Avastin®, a blocking monoclonal antibody against VEGF-signaling, has been shown to increase the overall survival for patients in a variety of cancers, including metastatic colon carcinoma,²¹⁴ NSCLC,²¹⁵ metastatic renal cell carcinoma,²¹⁶ and recurrent glioblastoma.^{217,218} On the contrary, Avastin® was accelerated approved for the use in metastatic breast cancer patients in 2008 but FDA revoked this approval in 2011 since two additional studies revealed no overall survival benefits.^{219,220} Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs), which inhibits inflammatory cells, has been shown to reduce the risk for gastrointestinal cancer.^{221–223} However, long-term use of NSAIDs may be problematic and result in dyspepsia, gastric bleeding, hypertension, renal failure, and cardiovascular diseases.²²⁴ One huge disappointment within the stromal targeted therapy area is, as mentioned before, the failure of MPIs.¹⁷¹ Because of this, the aim should be to find new drugs that target different aspects of the activated and abnormal stroma and combine them with cytotoxic therapies directed against the tumor cells. In that way the tumor will be treated as the heterogenic organ we now recognize it to be.

AIM OF THE THESIS

The aim of this thesis was to uncover factors that affect treatment response in HNSCC. Finding predictive markers for treatment response would enable a more individualized treatment plan for HNSCC patients.

Specific aims

- By genome-wide microarray analysis and bioinformatics processing identify a transcriptional profile for cisplatin resistance as well as identify key regulators which have impact on cisplatin treatment response.
- Investigate the functional importance of the EGFR ligands EGF, AR, and EPR in relation to proliferation and cetuximab sensitivity.
- Study how CAFs affect cetuximab treatment response in HNSCC cells.
- Investigate the crosstalk between CAFs and tumor cells that may underlie cetuximab resistance.
- Investigate gene expression differences between tumor cells co-cultured with their patient-matched CAFs and tumor cells cultured alone.

MATERIAL AND METHODS

Cell lines from Turku University

The HNSCC cell lines used in papers I and III were provided by Professor Reidar Grenman at Turku University, Finland. In paper I three cell lines were chosen for microarray analyses according to their sensitivity to cisplatin. A panel of 25 cell lines was used to evaluate the results. In paper III two larynx and two tongue cancer cell lines, all with an intermediate sensitivity for cetuximab, were selected. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine, penicillin, streptomycin, and fetal bovine serum (FBS) and used in passages 20-35.

Cell lines from Linköping University

Tumor biopsies have been collected from HNSCC patients at the ENT (ear, nose, and throat) department at Linköping University Hospital from January 2004 and on (approved by the ethical committee at Linköping University Hospital, Dnr 03-537) and from this material 25 HNSCC cell lines have been established as previously described.²²⁵ Cell lines were cultured in Keratinocyte-serum free medium (SFM) supplemented with penicillin, streptomycin, and 10% FBS and used in passages 10-25. Cells were screened periodically for mycoplasma contamination using DAPI staining and/or the ATCC Universal Mycoplasma Detection Kit.

Linköping HNSCC cell lines were used in papers II and IV. In paper II, the cell lines were selected according to their sensitivity to cetuximab, as well as the

location, histological grading, and TNM-status of the tumors from which they originate. In paper IV, five cell lines, to which patient-matched CAF-cultures were available, were used. These cell lines all originated either from tumors of the tongue or larynx.

Establishment of Linköping CAF-cultures

CAF cultures were established from the same tumor explants as the HNSCC cell lines and separated from the tumor cells by differential trypsinization. CAFs were cultured in DMEM supplemented with glutamine, penicillin, streptomycin, and 5% FBS (paper III) or Keratinocyte-SFM supplemented with penicillin, streptomycin, and 10% FBS (paper IV). Fibroblast origin was verified by positive immunofluorescent staining for vimentin and negative staining for cytokeratin.²⁰³

Seven different CAF cultures originating from tumors of the tongue, larynx, tonsil, gingiva, and buccal mucosa were used in study III. In paper IV, CAF cultures originating from five tumors, from which HNSCC cell lines were established, were used. For all experiments, CAFs were used at passages 2-6.

Assessment of intrinsic cisplatin- and cetuximab sensitivity

The intrinsic cisplatin sensitivity (ICS) of 39 Turku HNSCC cell lines have previously been determined by a clonogenic assay.²²¹ Cells were seeded in six-well plates and cisplatin was added to the cultures. Cells were cultured for another nine days before fixation in 4% paraformaldehyde (PFA), staining with Giemsa, and counting of colonies containing 32 cells or more.

For assessment of treatment sensitivity of the cell lines established in Linköping, the cells were seeded in 12-well plates and exposed to cisplatin or cetuximab.²²⁵ After incubation for nine days, the cells were fixed in 4% PFA and stained with 0.04% crystal violet in 1% ethanol. The stained colonies were solubilized in 1% SDS (sodium dodecyl sulfate) and optical density was measured using a Victor plate reader (Perkin-Elmer) at 550 nm.

Co-culture systems and collection of conditioned medium

Three different co-culture systems were used in papers III and IV in order to investigate if CAFs had any effects on the tumor cell growth, the response to cetuximab, or the tumor cell gene expressions. A transwell system (Figure 4A), using filter inserts with 0.4 μm pore size, through which cells are unable to pass, were used in experiments where cell-cell contact was not allowed. Tumor cells were first plated at the bottom of the well and were allowed to attach before CAFs were seeded on the filters, resulting in an approximate 1:1 ratio of CAFs to tumor cells. In this system, tumor cells and CAFs can only communicate through soluble factors. In the second system, in which cell-cell contact was allowed (Figure 4B), tumor cells and CAFs were co-cultured together in cell culture flasks. Tumor cell and CAF numbers were adjusted so that an approximate ratio of 3:1 was achieved by the end of co-culture. In the third system, CAF conditioned medium (CM) was added to tumor cell mono-cultures (Figure 4C). The amount of CM in the wells represented 25% of the total volume. CAF CM was collected from confluent cultures 72 h after medium change.

Figure 4. Three different co-culture systems were used in this thesis. **(A)** A transwell system, using filter inserts with 0.4 μm pore size polycarbonate membranes, which do not allow cell-cell contact between tumor cells and cancer associated fibroblasts (CAFs). **(B)** A co-culture of tumor cells and CAFs in cell culture flasks which allows for cell-cell contact. **(C)** Addition of fibroblast conditioned medium (CM), which contains CAF secreted soluble factors, to tumor cell mono-cultures.

Migration assay

In paper IV, a transwell migration assay was used in order to investigate the influence of CAFs on tumor cell migration. The tumor cells were stained with CellTracker™ Green and seeded with (allowing cell-cell contact) or without CAFs into filter inserts with an 8 μm pore size. The tumor cells were allowed to migrate for 48 h before they were collected and the fluorescence was measured at 485/535 nm with a Victor plate reader. A standard curve with cell numbers ranging from 5000 to 50000 was used to determine the number of migrating tumor cells.

Microarray and bioinformatics

In paper I microarray was used to study gene expression differences between one cisplatin sensitive cell line and two cisplatin resistant cell lines. The chip which was used in this paper (Affymetrix Human Genome U133 Plus 2.0) analyses the expression of 38 500 genes. By comparing the differentially expressed transcripts among the two resistant cell lines with the sensitive cell line, one can easier separate the characteristics of cisplatin resistance and study

the pathways. However, the amount of data retrieved from this analysis requires appropriate statistical and bioinformatics processing in order to obtain manageable results. The gene ontology tree machine (GOTM) analyses a set of genes and detects transcripts present in enriched groups based on their assigned gene ontology. Such analysis sheds light on the processes that are involved in cisplatin resistance and additionally facilitates the exclusion of transcripts of limited importance. Ingenuity pathway analysis (IPA) provides molecular networks from a reference gene list. This enables the identification of key regulators, i.e., molecules that interact with several other factors in a network, which in this case are involved in cisplatin resistance.

Microarray was also used in paper IV in order to compare the gene expression of tumor cells co-cultured with their patient-matched CAF and tumor cells cultured alone. The chip used in this paper (Affymetrix Gene Chip Human Exon 1.0 ST) analyses the expression on exon-level (i.e., enables to distinguish between different isoforms of a gene) on a whole-genome scale. In this paper R/Bioconductor packages were used as tool for the analysis and comprehension of the microarray results. IPA was used to investigate the association with important biological functions which may be related to the genes found to be differentially expressed in tumor cells co-cultured with CAFs when compared to tumor cells cultured alone.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis was used to determine the relative messenger RNA (mRNA) expression. The values were calculated by the comparative Ct method²²⁶ which presents the data as a fold-difference in expression level relative to a calibrator sample. Glyceraldehyde-3-phosphate dehydrogenase was amplified as an internal standard.

In paper I qPCR was used in order to verify the microarray results and to examine the expression of the genes of interest in a larger panel of cell lines. qPCR was also used in paper IV for verification of microarray results and to study the gene expression differences between tumor cells in co-cultures allowing cell-cell contact and co-cultures which do not. The qPCR method was used in papers II and III to verify gene knockdown by RNA interference. Moreover, in paper III the expression of a number of genes primarily involved in EMT, EGFR signaling, and treatment resistance were studied by multi qPCR.

Western blot

The semi-quantitative method western blot was used to examine the relative protein abundances in cell lysates. In paper III this technique was applied to analyze the expression of EGFR downstream effectors upon co-culture with CAF and cetuximab treatment. Unequal loading was adjusted by correlation of the protein bands to β -actin.

ELISA

ELISA is a quantitative method that can be used to determine the expression of a specific protein in a sample. In paper II, ELISA was used to verify gene silencing of AR, EGF, and EPR in HNSCC cells. In paper III, this method was also used to verify downregulation of MMP-1 and hepatocyte growth factor (HGF) expression in CAF CM, and to study the amounts of these proteins in CM from co-cultures as well as tumor cell and CAF mono-cultures. The amount of the detected proteins was determined using a standard curve constructed by plotting the mean relative fluorescence units (for MMP-1) or the mean absorbance (for AR, EGF, EPR, and HGF) for each standard against the concentration.

RNA interference

RNA interference is a method that specifically downregulates the expression of a particular gene. Short interfering RNAs (siRNAs) are short (<25 nucleotides) double-stranded duplexes which in the cells are unwound into two single-stranded RNAs (ssRNAs). One of the strands is degraded and the other is incorporated into the so-called RNA-inducing silencing complex. This complex targets and cleaves the mRNA that is complementary to the incorporated ssRNA and thus, gene silencing is achieved since the translation of targeted genes is interrupted.

In paper II siRNA-mediated knockdown of the EGFR ligands EGF, AR, and EPR was performed to study their effects on cetuximab treatment response and cell proliferation. siRNA transfection was also used in paper III to attain gene silencing of HGF and MMP-1 in order to clarify their role in CAF-induced cetuximab resistance.

Magnetic activated cell sorting

The magnetic activated cell sorting (MACS) method allows for separation of various cell populations based on their expression of surface antigens. The technique is based on magnetism and antibodies directed against a particular antigen. Cells expressing the specific antigen will be separated from cells which do not express the antigen. The separation of a subset of cells can be achieved either through positive selection, i.e., selection of the cells expressing the antigen of interest, or by negative selection, i.e., the antibody used is directed against surface antigens which are present on cells that are not of interest.

MACS was used in paper IV to separate tumor cells from CAFs after co-culture in culture flasks. Tumor cells were positively selected using magnetic beads

conjugated to antibodies directed against the epithelial marker EpCAM. These tumor cells were thereafter used in the microarray analysis.

Flow cytometry and fluorescence activated cell sorting

Flow cytometry is a method which can be employed for a variety of purposes, including cell counting, cell sorting, and protein detection. This technique is based on cells passing one by one through a laser beam where reflected and scattered light are measured. The reflected light gives information of the cell volume and the scattered light is correlated to shape of the nucleus, the amount and type of cytoplasmic granules, or the membrane roughness. One can also use fluorophore-conjugated antibodies that bind to a specific protein and thus estimate the amount of that protein. Fluorescence activated cell sorting (FACS) is one type of flow cytometry, which sorts cells using the information gained by the flow cytometry.

In paper IV, the FACS Aria flow cytometer was used in order to sort tumor cells expressing enhanced green fluorescent protein (EGFP). The EGFP expressing tumor cells were later co-cultured or not with CAFs with the purpose to investigate cell growth and cetuximab response. The number of EGFP expressing cells in co-cultures was determined by the flow cytometer, FACSCalibur.

Statistical methods

In paper I, the non-parametric Mann–Whitney U test was used to analyze possible association between MMP-7 and -13 expression and ICS.

In paper II, one-way ANOVA followed by t-tests with the Bonferroni adjustment was used to calculate differences between the groups.

In paper III, one-way ANOVA followed by Bonferroni or Dunnett's post-hoc tests was used to calculate differences between the groups.

In paper IV, Student's t-test was used to calculate differences between the groups.

For all statistical analyses performed in this thesis, p-values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

Results paper I

Cisplatin is the most common and effective chemotherapeutic drug used for treatment of locally advanced HNSCC but clinical resistance to the drug is a major problem. Since 70-80% of the patients that receive cisplatin do not respond to the treatment, but still experience the severe side-effects, we here aimed to find predictive markers of intrinsic cisplatin resistance(i.e., present at the time of initial treatment).

In order to identify differences between cisplatin sensitive and cisplatin resistant cells, we performed an unsupervised study using microarray analysis and 781 transcripts were found to be differentially expressed in both of the resistant cell lines compared to the sensitive cell line. To understand and manage the large amount of data retrieved from the microarray analysis the GOTM and IPA tools were used for enrichment of gene ontologies and network analysis, respectively. By applying the GOTM tool to the gene list 11 functional categories (Table 2, paper I) were found to be enriched in the two resistant cell lines versus the sensitive one. Using IPA seven molecular networks containing 20 key regulator genes, i.e., genes interacting with at least three altered transcripts were identified (Table 3, paper I). The top scoring network from the IPA analysis, in which the key regulators FN1, MMP-7, MMP-13, THBS1, and TIMP3 were identified, is shown in Figure 5.

Figure 5. The top scoring network from the IPA analysis in which FN1, MMP-7, MMP-13, THBS1, and TIMP3 were identified as key regulators of cisplatin resistance.

Of the 20 identified key regulators, six (APOE, CTNNB1, MMP-7, MMP-13, THBS1, and TIMP3) were found to be differentially expressed in the resistant cell lines compared to the sensitive. These were further selected for qPCR analysis, and for all genes except for TIMP3, the microarray result was verified. In order to further evaluate the importance of these key regulators APOE, CTNNB1, MMP-7, MMP-13, and THBS1 were selected for analysis of mRNA expression in a panel 25 HNSCC cell lines with known ICS. The cell lines were divided into two groups corresponding to high or low expression of the gene of interest, and the ICS was thereafter compared between the groups. It was demonstrated that a high MMP-7 expression significantly correlated to cisplatin

resistance ($p=0.0013$, Figure 2A, paper I) and that the MMP-13 expression showed a strong tendency to be associated with the cisplatin treatment response ($p=0.058$, Figure 2B, paper I).

Discussion paper I

Since resistance to cisplatin is a major problem in the clinic the search for predictive markers is crucial. In order to find markers predictive for cisplatin resistance one need to understand the molecular mechanisms that underlie chemoresistance. Therefore, in this study, we aimed to reveal the intrinsic dissimilarities between HNSCC cells that are resistant and cells that are sensitive to cisplatin.

The mechanisms underlying intrinsic cisplatin resistance may differ from those responsible for acquired resistance (i.e., adapted through repeated exposure to the drug) which is common during treatment. The acquired resistance is frequently due to decreased platinum accumulation, elevated drug inactivation by metallothionine and glutathione, and enhanced DNA repair activity⁹⁰ while the failure of apoptotic pathways^{92,227} such as the apoptotic signaling via Fas/Fas ligand (FasL) are commonly responsible for the intrinsic resistance.⁹³

Both MMP-7 and MMP-13 belong to the family of matrix metalloproteinases which includes at least 23 different members. Because of their influence on tumor progression and invasion MMPs are potential targets for anti-cancer treatments.¹⁷¹ Indeed, several MPIs have been developed through the years but the results from the clinical trials have been very disappointing. However, because of the design of these trials the knowledge about MMPs in carcinogenesis has improved; it is nowadays believed that MMPs play an important role in early cancer development as well as in the late stage.^{172,173}

MMP-13 belongs to the group of collagenases within the MMP family and their main action is to cleave interstitial collagens I, II, and III.¹⁷⁰ Studies have shown that MMP-13 frequently is expressed by primary HNSCC but rarely in normal tissue.²²⁸ This is well in line with the fact that MMP-13 was found to be upregulated during tumor growth and invasion, situations where rapid matrix turnover is needed. In addition, a high MMP-13 expression is associated with tumor aggressiveness and shorter overall survival in HNSCC and may have a prognostic value.²²⁹

Matrilysins, which includes MMP-7, is a group of MMPs that lack the hemopexin domain which is common to other MMPs.^{170,230} MMP-7 is one of the few MMPs which is synthesized and expressed in tumor cells themselves. MMP-7 is overexpressed in a majority of different cancer types, including HNSCC²³¹ and overexpression has also been shown to be associated with a shorter overall survival in this disease. MMP-7 is a very potent protease which cleaves many ECM components including elastin, type IV collagen, fibronectin, vitronectin, aggrecan, and proteoglycans.^{170,230} MMP-7 also plays an important role in the shedding of cell-surface molecules including tumor necrosis factor- α , HB-EGF, E-cadherin, β 4-integrin, and the Fas death receptor²³² and FasL.²³³ The shedding of E-cadherin is responsible for the increased migratory and invasive capacity of tumor cells, whereas the shedding of Fas and FasL by MMP-7 results in interrupted apoptosis and possibly resistance to cisplatin as the Fas/FasL pathway is known to contribute to cisplatin-induced cell death.²³⁰

Furthermore, MMP-7 expression has been found to correlate with EGFR activation.²³⁴ MMP-7 is also known to cleave HB-EGF resulting in cellular proliferation.²³⁰ Thus, an enhanced signaling through the EGFR may also contribute to MMP-7 associated cisplatin resistance.

This study proposes a role for MMP-7 in the evasion of apoptosis during cisplatin treatment and suggests that MMP-7 may be a predictive marker for cisplatin resistance in HNSCC. Since MMPs, including MMP-7, nowadays are believed to be important in early cancer development and that MMP-7 expression selects for cells with reduced sensitivity to cisplatin,²³⁵ the development of selective MMP-7 inhibitors, given at an early stage in combination with cisplatin, might render cells more sensitive to cisplatin.

Results paper II

Targeted therapy is a promising field in cancer management and such therapies are usually directed against growth factor receptors, such as EGFR, and their downstream signaling. The monoclonal EGFR antibody cetuximab has been approved for the treatment of locally advanced HNSCC but unfortunately only approximately 20% of the patients receiving this expensive treatment show disease stabilization and prolonged survival. Studies aiming at finding biomarkers that could predict the cetuximab treatment outcome in HNSCC patients are needed since there are no such markers available to date. In order to find biomarkers predictive of cetuximab response an increased knowledge about the EGFR signaling network is required. Therefore, in this study, we aimed to investigate the functional importance of the EGFR activating ligands EGF, AR, and EPR in relation to proliferation and cetuximab sensitivity in three tongue cancer cell lines; LK0412, LK0824, and LK0902.

The influence of EGF, AR, and EPR on tumor cell proliferation and cetuximab response was evaluated by the addition of recombinant human (rh) proteins or by siRNA-mediated downregulation of the endogenous ligand production.

The proliferative effect exerted by rhEGF differed among the three cell lines; an anti-proliferative effect was observed in the LK0412 and LK0824 cell lines, whereas an increase in the proliferative rate was seen in LK0902 (Figure 1A, paper II). A similar pattern was observed upon addition of rhEPR (Figure 1C, paper II) while rhAR did not exert any significant effect on cell growth in any of the three cell lines (Figure 1B, paper II). On the contrary, downregulation of endogenous AR caused reductions in tumor cell growth in all cell lines (Figure 2B, paper II). Also upon depletion of EGF a pronounced inhibition of proliferation was seen in all cell lines (Figure 2A, paper II).

One of the main findings in this study was that all three cell lines displayed increased cetuximab resistance upon the addition of rhEGF (Figure 3A, paper II). In contrast, only LK0824 showed increased resistance after stimulation with rhAR (Figure 3B, paper II) and rhEPR (Figure 3C, paper II). Interestingly, this cell line was the only one that showed an increased sensitivity to cetuximab after silencing of EGF (Figure 4A, paper II). Furthermore, the siRNA-mediated downregulation of AR sensitized LK0412 to cetuximab treatment while an increase in resistance was seen in LK0902 (Figure 4B, paper II). LK0824 was unaffected by the depletion of endogenous AR.

Discussion paper II

The tyrosine kinase cell surface receptor EGFR is overexpressed in approximately 30% of all human epithelial tumors¹⁴³ and in almost all HNSCC.¹⁴⁴ Since EGFR commonly is overexpressed and a high expression is associated with shorter overall survival and loco-regional failure, therapies aiming to prevent EGFR signaling have been developed. The monoclonal antibody cetuximab was approved for the treatment of locally advanced HNSCC by the FDA in 2006. Despite promising results in clinical trials the response rate in patients with HNSCC is only about 20%.^{107,110,116,117} In contrast to colorectal cancer, where KRAS mutation status is a well established predictive marker for cetuximab resistance,¹¹⁸ predictive markers for cetuximab treatment outcome are lacking in HNSCC. HNSCC is one of the most heterogeneous cancer types with tumors from many different locations (e.g., oral cavity, pharynx, larynx, and paranasal sinuses) and this might explain the difficulties in finding predictive biomarkers. Therefore, in this study, we selected a relatively homogenous cell line panel with three cell lines derived from mobile tongue and with similar TNM-status and histological grade (Table 1, paper II). Regardless of this the

results were diverse and highlight the problem with tumor heterogeneity and the complexity of the EGFR signaling network in cancer.

In this study, it was demonstrated that EGF and AR are important for tongue cancer cell proliferation and that EGF is a potential predictive biomarker of poor cetuximab response. Indeed, many studies have suggested EGFR ligands to regulate response to EGFR-targeted therapies.^{156–163,225,236} For example, it has been suggested that high expression of AR and EPR predict good response to cetuximab in KRAS wild-type colorectal cancers^{158,161,162} and in concordance with this, the development of acquired cetuximab resistance has been linked to the downregulation of AR and EPR.¹⁵⁶ Here, depletion of endogenous AR inhibited the tumor cell growth in all cell lines (Figure 2B, paper II) suggesting that downregulation of AR makes tumor cells less aggressive. However, the impact of AR depletion on the cetuximab response differed between the three cell lines (Figure 4B, paper II). While LK0902 became more resistant to cetuximab, which is in agreement with the above mentioned studies, LK0412 became more sensitive. Nevertheless, it has recently been shown that a high AR expression level correlates with poor response to cetuximab-docetaxel treatment in HNSCC patients.¹⁵⁹

EGF was found to induce resistance to cetuximab in all cell lines (Figure 3A, paper II). Moreover, depletion of endogenous EGF resulted in a significant increase in cetuximab sensitivity in the LK0824 cell line, and the same tendency was also seen in LK0412 (Figure 4A, paper II). This suggests that EGF is a potential predictive marker for cetuximab resistance in tongue cancer. Surprisingly, the mRNA expression of EGF was low and we were not able to detect any EGF in culture media from the cell lines tested. However, our results suggest that the low amount of EGF is sufficient to induce cetuximab resistance. Autocrine growth factor production of EGF might compete with blocking

antibodies for binding to the EGFR and thus reduce their effectiveness.²³⁷ The greater impact of EGF on the cetuximab treatment response could be explained by the fact that EGF has a much higher affinity to EGFR than AR and EPR.^{238,239} Interestingly, stromal cells were shown to have a higher expression of EGF than epithelial cells.²⁴⁰ Speculatively, stromal cells such as CAFs might be the main EGF producers within HNSCC tumors and may hence induce cetuximab resistance in a paracrine fashion.

Taken together, this study showed that EGF and AR are critical components of the EGFR signaling network that is required to maintain the full proliferative potential in tongue cancer cell lines and that EGF is a potential predictive biomarker of poor cetuximab response and a possible treatment target.

Results paper III

A tumor is not a homogenous mass consisting only of tumor cells but rather a complex network with tumor cells, fibroblasts, endothelial cells, pericytes, inflammatory cells, and ECM that interact in a multipart ecosystem. CAFs, which are one of the major components of the tumor microenvironment, are known to stimulate tumor growth and induce resistance to various anti-cancer treatments. For that reason we investigated the possible influence of CAFs on the cetuximab response in HNSCC cell lines.

During co-culture of four tumor cell lines with seven different CAFs in a transwell system (Figure 4A), a partial or full protection against the effect of cetuximab on the tumor cells was observed (Figure 1, paper III). In the UT-SCC-9 cell line cetuximab treatment even stimulated cell growth in the presence of CAFs (Figure 1A, paper III). However, this stimulation was not seen for UT-SCC-24A (Figure 1B, paper III), UT-SCC-19A (Figure 1C, paper III), or UT-SCC-76A (Figure 1D, paper III). Interestingly, the stimulation of UT-SCC-9 cell growth during cetuximab treatment was not accompanied by CAF-induced changes in the tumor cell proliferation rate in the absence of cetuximab (Figure 2A, paper III). Conversely, the other three cell lines, in which CAFs showed a more modest protection from cetuximab, were all growth stimulated in the presence of CAFs (Figures 2B-D, paper III).

To find out whether the increased resistance to cetuximab was due to CAFs specifically interfering with the interaction between cetuximab and EGFR or if they counteracted the action of EGFR-targeting therapies in general, the cells were treated with the EGFR-selective tyrosine kinase inhibitor gefitinib. Similar results were found for gefitinib as for cetuximab (Figure 3, paper III) suggesting that CAFs affect EGFR inhibition in general.

When medium conditioned by CAFs was added to the cells (Figure 4C) a similar protection from cetuximab treatment was seen as during co-culture with CAFs (Figures 4A and B, paper III). These results suggest that treatment resistance is mediated by CAF-derived soluble factors. In addition, by size-fractionation of the CM it was shown that soluble factors larger than 50 kDa are likely to be responsible for the protective effect (Figure 4D, paper III).

The cetuximab resistance observed in co-cultured tumor cells could not be explained by changes in the expression or phosphorylation status of EGFR within tumor cells (Figures 5A and B, paper III) or by secretion of HGF from CAFs (Figure 6, paper III). In an attempt to identify the soluble factors responsible for the increased resistance to cetuximab in tumor cells, we performed a mass spectrometry analysis in which CM was compared with complete growth medium. However, we were unable to get any conclusive results from this analysis. The lack of results can be explained by the presence of high-abundance serum proteins in the CM, which may have obscured any low-abundance factors present. Unfortunately CAFs could not be cultured in SFM without showing reduced viability. Therefore we instead performed a multi qPCR in order to study expression differences between mono- and co-cultured tumor cells in a panel of genes. From this analysis we observed an elevated expression of MMP-1 in co-cultured tumor cells, and this was later found to be true also for co-cultured CAFs (Figure 7A, paper III). The CAF-induced cetuximab resistance was significantly decreased when using an MMP inhibitor; (Figure 7C, paper III) however, downregulation of MMP-1 in CAFs or in tumor cells did not increase the sensitivity to cetuximab (Figures 7D and E, paper III). This may indicate that several CAF-regulated MMPs cooperate in the induction of resistance.

Discussion paper III

Cancer research has for a long time been focused mainly on the cancer cell and its molecular changes; however, it is now accepted that the tumor microenvironment contributes to cancer progression and may influence the response of different cancer types to various anti-cancer treatments. CAFs, which are the most abundant cell type of the tumor stroma, have been shown to modulate the drug sensitivity in cancers of epithelial origin. Indeed, CAFs were demonstrated to induce resistance to tamoxifen in breast cancer,^{205,208} paclitaxel and gefitinib in NSCLC,^{206,207} and to gemcitabine and radiotherapy in pancreatic adenocarcinoma.²⁴¹ Here, we show for the first time that CAFs also induce resistance to cetuximab. However, the soluble factors responsible for this increased resistance to cetuximab in HNSCC are yet to be identified. Interestingly, CAF-secreted HGF has been suggested to be the causing factor for resistance to gefitinib²⁰⁶ and to an irreversible EGFR tyrosine kinase inhibitor²⁴² in NSCLC. Clinical trials combining c-Met inhibitors with EGFR tyrosine kinase inhibitors are ongoing.^{243,244} In our hands, addition of rhHGF to tumor cell monocultures confirmed that HGF can induce treatment resistance (Figure 6B, paper III). Still, even though endogenous HGF was depleted in CAF-cultures the CM collected from these cultures still protected the tumor cells from cetuximab (Figure 6C, paper III). This excluded HGF to be the soluble factor contributing to the CAF-induced cetuximab resistance in HNSCC cells. However, CAFs may derive from many different subsets of cells and are likely to display functional differences depending on their origin¹⁹³ thus, different CAF-secreted factors may be important for treatment sensitivity in diverse cancer types.

We performed a multi qPCR to study differences in gene expression between mono- and co-cultured tumor cells in order to identify the soluble factors

responsible for the increased resistance to cetuximab. From this analysis tumor cells co-cultured with CAFs were found to have an upregulated expression of MMP-1 (Figure 7A, paper III). In addition, co-cultured CAFs also had a higher expression of MMP-1 than CAF mono-cultures which propose that factors originating from both CAFs and tumor cells are responsible for the resistance to cetuximab. Interestingly, an inhibitor of MMP-1 reduced the protective effect of CAF-CM (Figure 7C, paper III) but the CAF-induced cetuximab resistance was not diminished by downregulation of endogenous MMP-1 in either CAFs or tumor cells (Figures 7D and E, paper III). The MMP-1 inhibitor used could to some extent also block MMP-2, -3, -7, and -13, indicating that a MMP other than MMP-1 may mediate the resistance, or that several CAF-regulated MMPs cooperate in the induction of drug resistance. Hence, in a family of approximately 23 members it is unlikely that only one MMP could have significant impact on cetuximab treatment resistance.

Since our results suggest that factors associated with both CAFs and tumor cells influence the outcome of EGFR-targeted therapy this highlights the importance that anti-cancer therapies should target both stromal cells and tumor cells. However, it is very difficult to find the molecules that transmit signals between tumor cells and stromal cells since the nature of these molecules is largely obscure and this complicates the development of therapies targeting the tumor stroma. High-throughput screening of tumor cells and CAFs may be a helpful tool in understanding the complex molecular crosstalk between stromal cells and tumor cells and ultimately in the discovery of factors affecting treatment resistance in tumor cells.

In this study we identify a novel CAF-dependent modulation of cetuximab sensitivity and suggest that inhibiting MMPs may improve the effects of EGFR-targeted therapy.

Results paper IV

In paper III we showed that the increased resistance to cetuximab was due to secreted factors from CAFs but unfortunately we were unable to identify the specific factor/factors responsible for this. Therefore, in this study we performed a microarray analysis in order to compare gene expression differences between tumor cells co-cultured with their patient-matched CAFs and tumor cells cultured alone, in an attempt to identify the causative soluble factors.

Upon co-culture with CAFs, a thousand genes or more were deregulated in each individual tumor cell line tested. More importantly, 58 protein coding genes ($Q < 0.05$) were found to be differentially expressed in all five co-cultured tumor cells. Of these 58 genes, nine were upregulated by ≥ 1.5 -fold (Table II, paper IV) while 35 were found to be downregulated by ≤ 0.67 -fold (Table III, paper IV). Interestingly, many of the deregulated genes have earlier been associated with EMT. Five of the differentially expressed protein coding genes (COL1A2, GREM1, MMP-7, POSTN, and VIM) were selected for verification by qPCR and for COL1A2, GREM1, POSTN, and MMP-7 a change in expression upon co-culture with CAFs was verified in all five cell lines (Figures 3A-C, E, paper IV). The decreased expression of VIM could be verified in four out of the five cell lines (Figure 3D, paper IV). Furthermore, these changes in expression levels were all found to be dependent on cell-cell contact (Figure 4, paper IV) since they could only be detected in co-cultures allowing cell-cell contact (Figure 4B) and not in the transwell system (Figure 4A).

The influence of CAFs on tumor cell proliferation, cetuximab response, and migration was also investigated and gave varying results (Figure 5, paper IV) e.g., a slight protective effect from cetuximab was observed in one of the cell

lines upon co-culture with CAFs while the opposite was seen in another (Figure 5B, paper IV).

Discussion paper IV

As nowadays accepted, a tumor is a heterogeneous and structurally complex tissue which does not consist of only malignant cells but also of fibroblasts, endothelial cells, pericytes, and inflammatory cells, which together with the ECM constitute the tumor stroma. CAFs are the major cellular component of the tumor stroma and they have been shown to stimulate tumor growth,^{188,194,195} EMT,^{184,186,196,197} invasion,^{198,199} and metastasis^{196,200} and to influence treatment response, by secretion of soluble factors.^{203–211} The detection of the soluble factors that are responsible for the influence on treatment response could lead to the development of new treatment strategies. However, the crosstalk between cancer cells and CAFs are still not completely understood and the nature of the soluble factors is largely obscure.

In this study, we show that CAFs affect the gene expression in tumor cells and that this is dependent on cell-cell contact. This might be due to both juxtacrine and paracrine signaling in co-cultures allowing cell-cell contact. In addition, the local concentration of soluble factors might be higher when the two cell types are in close contact which may be required in order to induce gene expression changes in the tumor cells.

In paper III we showed that CAFs induced resistance to cetuximab in HNSCC cells, however; in this study the results differed among the three cell lines i.e., one cell line became more resistant to cetuximab whereas one showed higher sensitivity (Figure 5B, paper IV). The discrepancy between the results might be due to the difference in the individual ability of tumor cells to respond to CAF-signals, as seen in both paper III (Figure 2) and paper IV (Figure 5). As HNSCC

cells are highly heterogeneous they will probably respond differently toward CAF-signals. Therefore, targeting one aspect in the complicated crosstalk between tumor cells and CAFs will most likely not work. Hence, an increased understanding of the crosstalk between cancer cells and CAFs is of critical importance in order to find different treatment targets within the tumor stroma which can be used in combinations with standard treatments.

Taken together, we here show that CAFs induce multiple gene expression changes in their patient-matched tumor cells, and that 58 of them are common among all five tumor cell lines investigated. A number of these deregulated genes have been implicated in the EMT process. Furthermore, our data shows that tumor cells and CAFs need to be in close contact for at least some of these changes to occur.

CONCLUCIONS

The following conclusions can be drawn from the results presented in this thesis:

- High expression of MMP-7 is significantly correlated to intrinsic cisplatin resistance.
- High expression of MMP-13 shows a strong tendency to affect cisplatin treatment outcome.
- EGF is a potential predictive biomarker of poor cetuximab response and a possible treatment target.
- CAFs induce resistance to cetuximab treatment through the secretion of soluble factors.
- MMPs are partly responsible for the observed CAF-induced resistance to cetuximab.
- CAFs induce multiple gene expression changes in tumor cells, some of which are associated to EMT.
- The CAF-induced gene expression changes of COL1A2, GREM1, MMP-7, POSTN, and VIM is dependent on cell-cell contact.
- The tumor cell response to CAF-signals varies between individuals.

FUTURE PERSPECTIVES

HNSCC is one of the most distressing human cancer types and two-thirds of the patients have advanced disease with lymph node metastasis at diagnosis. Since patients with HNSCC tends to be diagnosed at a late stage, which usually means a poorer prognosis than for early stage tumors, efforts to achieve earlier diagnoses should be made. Late diagnosis is probably due to lack of knowledge about the disease and ignorance of symptoms, which delay the visit to a physician. Since most people go to the dentist more often than to the doctor, information on HNSCC from their dentist or dental hygienist may make people visit a physician at an earlier time point.

HPV-positive HNSCCs have grown strong in Sweden in recent years. A preventive act for this would be to expand the HPV-vaccination program to also include boys, especially since it has been shown that men have a higher rate of HPV-positive tumors. Today the HPV-vaccination aims to reduce the incidence of HPV-induced cervix cancer in women.

Besides preventive actions, it is very important to find improvements for the treatment regime that can be used in the clinic. The field of oncology has now entered the era of personalized medicine where treatments can be individually selected for each patient. Clinically useful predictive biomarkers of treatment outcome have been found for colorectal and breast cancers but is still lacking for HNSCC. This is likely due to the high heterogeneity within this group of tumors, which makes them act and respond very differently to treatments. Indeed, we have in the studies in this thesis seen that the response of HNSCC

tumor cells to CAF-signals varies greatly. In addition, even though we chose a more homogeneous panel of cell lines in one of our studies, i.e., tongue cancer cell lines with similar TNM-stage and histological grading, they still displayed very different responses to stimuli with EGFR ligands. In the future, the search for predictive markers of treatment response in HNSCC should aim to divide this group of tumors even further, not only depending on location of the tumor. Smokers/non-smokers (or other tobacco products), HPV-positive/HPV-negative, males/females, histological grades, and TNM-stages are examples of further groupings. However, it is necessary to collaborate with other clinics, as this will otherwise result in too small patient groups. We have started collaborations with the ENT clinics in Helsinki, Turku, and Stockholm, which enables us to verify our results obtained in *in vitro* studies in a larger group of patients subdivided according to tumor location, smoking habits, and HPV-status etcetera.

Finally, it would be very interesting to further investigate the functional importance of the deregulated genes found in paper IV, in relation to tumor cell proliferation, migration, and response to cetuximab.

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