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The impact of Survivin, WRAP53 β , and Hypoxia on treatment response in Head and Neck Cancer

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To my beloved FREDRIK,
and my beloved family

“If we knew what it was we were doing, it
would not be called research, would it?”

- Albert Einstein

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ABSTRACT

Squamous cell carcinoma (SCC) is the most common histological type of cancer in the head and neck region and arises in the epithelial mucosa of the upper aerodigestive tract. Approximately one and a half million people are living with the diagnosis. Despite efforts in prevention and advances in treatment, the 5-year survival rate still lies around 60%, and recurrences and second primary tumors remain a problem. Moreover, treatment responses vary from patient to patient, highlighting the need for individually tailored treatments. To make this possible, biomarkers predicting treatment outcome are needed to better guide treatment decisions.

The aim of this thesis was to evaluate the expression of certain proteins and the frequency of certain SNPs (Single nucleotide polymorphisms) in tumor biopsies and cell cultures of head and neck squamous cell carcinomas (HNSCC), and to explore their potential as biomarkers for treatment outcome. Furthermore, we aimed to study the impact of hypoxia on treatment response, epithelial-to-mesenchymal transition (EMT), and induction of cancer stem cells (CSC).

In papers I and II, we investigated two proteins, survivin and WRAP53 β , using immunohistochemistry (IHC) in tumor biopsies from 40 patients categorized as Non-responders or Responders to radiotherapy. High expression of survivin and nuclear expression of WRAP53 β were significantly more prevalent in the Responder group. The combination of these two factors correlated strongest to overall survival, but not to a significantly higher extent compared to survivin alone. Moreover, when examined separately, a high percentage of p53-stained cells and the presence of the SNP FGFR4 Gln388Arg correlated to improved overall survival, whereas the SNP XPD Lys751Gln was associated with worse overall survival. The latter three showed no significant correlations to radiotherapy response. In paper III, the two

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most promising proteins identified in papers I and II were analyzed in a study cohort of 149 tumor biopsies of glottic laryngeal SCC, categorized as T2N0-T3N0. In this patient group, no significant associations between survivin expression and survival could be found. However, expression of cytoplasmic WRAP53 β was significantly linked to worse disease-free-survival (DSF) compared to nuclear WRAP53 β or negative staining for WRAP53 β . Positive expression of p16^{INK4a} was found in 7% of the tumors. The prevalence of p16^{INK4a} was higher in younger patients (<60) and associated with absence of recurrence and longer DSF.

In paper IV, five HNSCC cell lines were cultured in normoxic (20% O₂) and hypoxic (1% O₂) conditions and changes in treatment response, EMT profile, and expression of CSC markers were examined. As expected, hypoxia induced EMT and to a certain extent expression of CSC markers. Silencing of the hypoxia-inducible-factor-1 α (HIF-1 α) only partly reversed these effects, suggesting that other mechanisms are involved. Whereas most cell lines became more resistant to treatment in hypoxia, one cell line (LK0412) became more sensitive to cetuximab-treatment in hypoxia, an effect that was revoked by depletion of HIF-1 α , suggesting a possible sensitizing effect of HIF-1 α to cetuximab-treatment.

Taken together, WRAP53 β appears to be a promising biomarker candidate for treatment outcome in HNSCC, but further evaluation especially on the subcellular localization of WRAP53 β is required. Even though the role of survivin in radiotherapy response in glottic SCC seems to be insignificant, it might have a more important role in other HNSCC subsites. As far as the effects of hypoxia, it appears that hypoxia might have a sensitizing effect on cetuximab-treatment in certain cases, which seems to be HIF1- α –dependent. Further studies are required to clarify the importance of this observation.

POPULAR SCIENCE SUMMARY

The term Head and Neck Cancer summarizes cancer that arises anywhere in the head and neck region, most commonly on the lips, in the mouth, and in the throat. Long-time use of harming agents like tobacco and alcohol are the main risk factors and lead to changes in the cells, eventually (after many years) resulting in uncontrolled cell growth, cancer. Infection with human papilloma virus (HPV) is another important cause and has become more common, probably because of changes in sexual behavior. Depending on where the cancer is located, patients may have difficulties speaking, swallowing, or breathing. Furthermore the cancer itself can be mutilating, causing a great deal of suffering. The treatment options, which include surgery, radiation, and chemotherapy, are also hard for the patients and cause various side-effects. Unfortunately, not all patients will be helped by these treatments and some patients even get worse. Possible explanations to this variation in treatment response are variations in genes (mutations) and protein expression within the tumor. Increasing our knowledge about factors within the tumor which may predict treatment response, is vital in order to achieve optimal, individualized treatment plans for each patient. Exploring such factors as potential biomarkers for treatment outcome was one of the main aims of this thesis. We found that the proteins survivin and WRAP53 β might be promising biomarkers for radiotherapy, but more studies are required to really understand the relation of these factors to treatment effect. Another aim of this thesis was to examine the effects of low oxygen levels (hypoxia) on treatment response. Hypoxia can exist in areas of the tumor, and it is known that treatments are generally less effective in hypoxic tumors. In our study, we found that hypoxia may not be a negative factor in all tumors and might even promote tumor sensitivity to certain treatments. However, this conclusion is based on very modest findings and no general assumptions can be made. Further studies are needed to explore predictive biomarkers and to better understand the complex role of tumor hypoxia for treatment response.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numbers I – IV:

- I. Lovisa Farnebo, **Katharina Tiefenböck**, Anna Ansell, Lena K. Thunell, Stina Garvin and Karin Roberg
Strong expression of survivin is associated with positive response to radiotherapy and improved overall survival in head and neck squamous cell carcinoma patients
Int. J. Cancer: 133, 1994-2003 (2013).
- II. Stina Garvin, **Katharina Tiefenböck**, Lovisa Farnebo, Lena K. Thunell, Marianne Farnebo and Karin Roberg
Nuclear expression of WRAP53 β is associated with a positive response to radiotherapy and improved overall survival in patients with head and neck squamous cell carcinoma
Oral Oncology 51 (2015) 24 – 30.
- III. **Katharina Tiefenböck-Hansson**, Aaro Haapaniemi, Lovisa Farnebo, Björn Palmgren, Jussi Tarkkanen, Marianne Farnebo, Eva Munck-Wikland, Antti Mäkitie, Stina Garvin and Karin Roberg
WRAP53 β , survivin and p16^{INK4a} expression as potential predictors of radiotherapy/chemoradiotherapy response in T2N0-T3N0 glottic laryngeal cancer
Oncol Rep. 2017 Oct;38(4):2062-2068.
- IV. Emilia Wiechec, **Katharina Tiefenböck-Hansson**, Lisa Alexandersson, Jan-Ingvar Jönsson and Karin Roberg
Hypoxia Mediates Differential Response to Anti-EGFR Therapy in HNSCC Cells
Int J Mol Sci. 2017 May; 18(5): 943.

ABBREVIATIONS

AF	Accelerated fractionation
ARTSCAN	Accelerated radio therapy of squamous cell carcinoma in the head and neck
CAIX	Carbonic anhydrase IX
CPC	Chromosomal passenger complex
Crm1	Chromosome region maintenance 1
CSC	Cancer stem cells
CT	Chemotherapy
DFS	Disease-free survival
DIABLO	Direct inhibitor of apoptosis-binding protein with low pI
DNA	Deoxyribonucleic acid
DSS	Disease-specific survival
EBV	Eppstein-Barr-virus
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ENT	Ear, nose, and throat
ERK 1/2	Extracellular signal-regulated kinases 1/2
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGFR4	Fibroblast growth factor receptor 4
FN1	Fibronectin 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gy	Gray
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HNSCC	Head and neck squamous cell carcinoma
HPV	Human Papilloma Virus
HPV-	HPV negative
HPV+	HPV positive
HRE	Hypoxia response elements
HRP	Horseradish peroxidase
IAP	Inhibition of apoptosis
ICmabS	Intrinsic cetuximab sensitivity
ICS	Intrinsic cisplatin sensitivity
IHC	Immunohistochemistry
IMRT	Intensity-modulated radiotherapy
INCENP	Inner centromere protein
IR	Intrinsic radiosensitivity
IRS	Immunoreactive score
JAK	Janus kinase
ISCC	Laryngeal squamous cell carcinoma
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MET	Mesenchymal to epithelial transition
mRNA	Messenger RNA
NES	Nuclear export signal
NR	Non-Responders
O ₂	Oxygen
OD	Optical density

ABBREVIATIONS

OPSCC	Oropharyngeal squamous cell carcinoma
OS	Overall survival
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
pEGFR	Phosphorylated EGFR
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PLC γ	Phospholipase C γ
pO ₂	Partial pressure of oxygen
pRb	Retinoblastoma protein
R	Responders
RFLP	Restriction fragment length polymorphism
RFS	Relapse-free survival
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPN	Ribonucleoprotein
RT	Radiotherapy
RT-qPCR	Reverse transcriptase –quantitative polymerase chain reaction
scaRNA	small cajal body specific RNA
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
Ser20	Serine 20
siRNA	small interference RNA
SMA	Spinal muscular atrophy
SMAC	Secondary mitochondria- derived activator of caspase
SMN	Survival of motor neuron
SNP	Single nucleotide polymorphism
snRNP	small nuclear ribonucleoprotein
STAT3	Signal transducer and activator of transcription 3
TCAB1	Telomerase cajal body protein 1
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
Thr34	Threonine 34
TNM	Tumor-node-metastasis
VEGF	Vascular endothelial growth factor
WDR79	WD repeat domain 79
WHO	World Health Organization
WRAP53	WD40 encoding RNA antisense to p53
XIAP	X-linked inhibitor of apoptosis protein
XPC	Xeroderma pigmentosum complementation group C
XPD	Xeroderma pigmentosum complementation group D
XRCC	X-ray repair cross-complementing

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1 INTRODUCTION

Cancer is a leading cause of morbidity and mortality worldwide (1) and the majority of the population has come or will come in contact with it, either as patients, relatives, friends or professionals. Cancer causes a lot of personal suffering and is an enormous economic burden to society, a burden that is expected to increase due to both the growth and aging of the population. The term cancer originated from the greek word *karkinos* and was minted by Hippokrates to describe big superficial tumors (2, 3). Other terms are malignant tumors or malignant neoplasms. Siddharta Mukherjee called it „The Emperor of all maladies“, as it is an old disease that has effected and concerned patients, physicians, and researchers for hundreds to thousands of years. In his book he describes the history of this disease and the dedication of physicians and researchers who have made it a goal in their lives to find the ‘ultimate cure for cancer’ (3). Through time, there have been many theories as to what the causes of cancer are. For instance, it was believed that cancer was the expression of an imbalance in the body fluids or caused and spread by a parasite (2). Nowadays cancer is described as “a group of diseases involving abnormal cell growth with a potential to invade or spread to other parts”(4). Due to huge advances in research on the molecular level in the 20th century we have been able to gain valuable insights into some of the underlying mechanisms of cancer. Still, even though some cancer types can now be cured, we do not fully understand cancer yet, and despite extensive research, no ultimate cancer cure has been found.

1.1 Head and Neck Squamous Cell Carcinoma (HNSCC)

This thesis focuses on squamous cell carcinoma arising in the head and neck region (Figure 1, provided and printed with the permission of Lovisa Farnebo (5)).

Figure 1. Regions of origin of HNSCC. The highlighted regions are the most common and represent the localization of tumors studied in this thesis.

The sites of the lip, oral cavity, oro-/ hypopharynx, and larynx are most commonly affected and constitute together the seventh most common cancer worldwide. 1,450,000 people are living with the diagnosis HNSCC (5-year prevalence), approximately 600,000 are newly diagnosed every year, and around 325,000 HNSCC-related deaths occur annually (6). Moreover, when including the salivary glands, the nasopharynx, and the upper 2/3 of the esophagus (which are cancers of the upper aerodigestive tract) it becomes the third most common cancer in men, the seventh in women (7).

1.1.1 Etiology and risk factors

Histologically, more than 90% of head and neck cancers are squamous cell carcinomas originating from epithelial cells of the mucous membrane. Multiple environmental, behavioral, and biological risk factors are involved in the development of HNSCC. The main risk factors are lifestyle-dependent and involve alcohol and various forms of tobacco (smoking or chewing). Those two main risk factors have a known synergetic effect (8, 9). Other behavioral risk factors are micronutrient deficiency (=low intake of fresh fruit and vegetables containing antioxidants) (10) and poor oral hygiene (11, 12). Viruses constitute the biological risk factors: human papilloma virus (HPV) has been mainly linked to cancer in the oropharynx and tonsils (13, 14) and Epstein-Barr-Virus to nasopharynx cancer (15). Environmental risk factors include UV light (especially lip-cancer), in- and outdoor air pollution, and occupational exposures to radiation or chemical carcinogens (inhaling of wood dust, asbestos, manufacturing of textiles and leather)(16).

1.1.2 The patient

Traditionally, HNSCC occurs in patients in the fifth decade of life and above, with a higher incidence in men than women and a clear connection to traditional risk factors (alcohol, smoking). However, in recent years there has been a notably increase of HNSCC in patients younger than 40 years (17, 18). This development was highlighted by the absence of traditional risk factors in these younger patients and led to the identification of high-risk HPV as a cause of oropharyngeal cancer, and finally to the distinction between HPV⁺ (HPV-positive) and HPV⁻ (HPV-negative) oropharyngeal tumors as two clinically different entities (17). HPV⁺ OPSCC (oropharyngeal SCC) is associated with a more aggressive phenotype, but also with a better response to radiotherapy and patient survival compared to HPV⁻ tumors (19).

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1.1.3 The symptoms and prognosis

Depending on the location of HNSCC, symptoms vary and the quality of life in these patients can be highly affected. Symptoms are caused by destruction of the anatomic structures and impairment of the organ functions by tumor growth. For instance, in tongue cancer speech and swallowing are impaired. In larynx cancer, hoarseness and breathing problems occur. Due to tumor growth the symptoms can become life-threatening, for instance by blocking the upper airway or by massive bleeding from large vessels. In the beginning, symptoms can be very subtle and patients often seek medical attention when the tumor has grown into advanced stages. At this time aggressive treatment with harsh side-effects is often unavoidable and the prognosis is poor. Although huge advances have been made in diagnostic techniques and in treatment modalities, the survival rate still lies around 60%.

1.2 Treatment

When a patient is diagnosed with HNSCC, the individual's treatment options are discussed by a multidisciplinary board, involving specialists in the fields of ENT, oncology, radiology, and pathology. For decision-making the TNM-classification (T=tumor size, N=nodal status, M=metastasis; clinically and radiologically determined), the primary site, the histological grade (low, medium, high), and the patient's health status are taken into account. The main standard treatments are surgery and radiotherapy, either alone or in combination. Generally, low stage tumors (stage I-II) are treated with single modality therapy, either surgery or radiotherapy alone, whereas high stage tumors (III and IV) are treated with a multimodality approach including also chemotherapy and molecular targeted drugs (17). Treatment intention is either curative or palliative. In recurrences, the former treatment and the current patient performance status have to be considered in regards to further therapeutic options. Since HPV-status has emerged as an important prognostic marker in HNSCC it now has to be taken into account for treatment decision in oropharyngeal cancer, as de-intensification of treatment might be an option (19).

1.2.1 Surgery

Surgery is the treatment of choice in tumors that are resectable with good tumor-free margins without venturing the organ function severely, e.g. low stage (T1) tongue cancer or glottic laryngeal cancer. Surgical options are open surgery with simple tumor excision and primary wound closure or, in certain cases, secondary wound healing (e.g. in tongue cancer), or advanced excision with reconstruction and local pedicled- or free microvascular flaps. Other surgical options are minimal invasive transoral laser surgery or transoral robotic surgery. Limitations of surgery are the preservation of organ function and avoidance of severe cosmetic deformity. Both impaired organ function (e.g. dysphagia, hoarseness) and cosmetic deformity cause a tremendous decrease in quality of life (17). Another limitation is the relation to surrounding tissue, like perineural or perivascular growth, making the tumor inoperable and oncological treatment the only option.

1.2.2 Radiotherapy

Radiotherapy (RT) is the other main treatment regime in HNSCC and is an option both in the curative and palliative setting. In early stage tumors, e.g. laryngeal cancer, tonsil cancer, or tongue base cancer, it can be given as a single therapy, whereas it is part of a multimodality approach in locally advanced tumors (e.g. prior or subsequent to surgery; concurrent with chemotherapy or molecular targeted anticancer therapy). The ionizing radiation induces DNA damages either indirectly by generating free oxygen radicals or by directly interacting with DNA (20). This results in initiation of cellular responses like cell cycle arrest, DNA repair, senescence, apoptosis or mitotic catastrophe, the latter being the major cell death mechanism in solid epithelial tumors (21). The greatest disadvantage and limitation of RT is treatment toxicity. Acute and late side-effects like mucositis, skin rashes, dysphagia, xerostomia, and osteoradionecrosis all cause suffering and reduce quality of life. Therefore the patient's performance status, previous radiation, and the proximity of risk organs to the tumor mass have

INTRODUCTION

to be considered thoroughly before a treatment decision is made, as they all play a role in the tolerance of treatment toxicity and grade of acute and late side-effects. Different techniques have been developed to minimize the damage on risk organs and normal tissue. Fractionation of the total dose is based on the fact that normal cells proliferate slower than cancer cells and have time to repair damage before replication (20). Conventional fractionation is given with 2 Gy once daily, 5 days/week for 6.5 weeks with a total curative dose of 68 Gy. Altered fractionation regimes (hyperfractionation, accelerated fractionation) have been developed to increase local control of the tumor with a cost of increased acute toxicity. Several clinical trials have been performed to compare different treatment techniques in locally advanced head and neck cancer (22). Parsons *et al* concluded that hyperfractionated radiotherapy (a reduced fraction given twice daily in a similar time period with an increased total dose) was superior to conventional RT, with greater acute side-effects but less late side-effects. Accelerated fractionation (AF: two fractions daily, with the same total dose but a shorter delivery period) showed no significant difference regarding locoregional control or survival compared to conventional RT in the 5-year report of the ARTSCAN study (23). Acute morbidity was significantly worse in patients treated with AF but there was no difference in late side-effects (23). Improvements in dose delivering techniques have also been done in regard to sparing risk organs and normal tissue. By intensity-modulated radiotherapy (IMRT), the tumor mass is treated with a higher dose than the healthy surrounding tissues and risk organs, thus minimizing side-effects without venturing treatment outcome (24).

1.2.3 Chemotherapy

In HNSCC chemotherapy (CT) is given in combination with radiotherapy, either with or without surgery. The cytostatic drugs are given intravenously and function either by blocking the synthesis of DNA or by attacking the integrity of DNA. Chemotherapy can be given as induction or neoadjuvant CT (= before radiotherapy), as concomitant CT (= at the same time)

or as adjuvant CT (=after radiotherapy). Given in a neoadjuvant setting, the aim is to shrink the tumor, allowing for easier treatment procedures with surgery or radiation. CT given concomitantly or adjuvantly amplifies the treatment effect, improving locoregional control and patient survival, and decreasing the risk of recurrence. The addition of chemotherapy has been shown to increase survival 4 - 9%, varying between different tumor sites, the largest effect with concomitant CT (25, 26). Concomitant chemoradiotherapy with the platinum-based drug cisplatin is a standard treatment in locally advanced HNSCC. Other cytostatic drugs used in HNSCC are 5-Fluorouracil (5-FU) and taxanes (e.g. docetaxel). The combination of these three drug types are used in induction CT and can be considered in selected patients with a high-risk of distant and local failure (26, 27). The limitations of chemotherapy are the severe side-effects (acute and late toxicity) and thus reduced tolerance in elderly patients and in patients with comorbidities.

1.2.4 Targeted Cancer Therapy

Targeted cancer therapies use drugs that interfere with specific molecules (=specific targets) involved in cellular processes such as cancer cell proliferation, progression, invasion, and survival. The treatment interference leads to inhibition of cancer growth and spread. Different types of targeted therapies exist, but so far the only FDA-approved targeted cancer therapy in HNSCC is Cetuximab.

1.2.4.1 Cetuximab

Cetuximab is a monoclonal antibody that targets the epidermal growth factor receptor (EGFR). With an approximately 10-fold higher affinity to EGFR compared to endogenous ligands, cetuximab binds EGFR and inhibits activation of EGFR downstream signaling. Moreover, it induces internalization and thereby downregulation of EGFR (28). In *in vitro* experiments cetuximab has been shown to inhibit cell proliferation and to induce apoptosis as well as to enhance radiosensitivity (29). Since then, cetuximab has become a part of the treatment strategy

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in patients with locally advanced HNSCC. Given concurrently with radiotherapy it has proven to have beneficial effects compared to radiotherapy alone (30) with an improved overall survival of 20 months. However, cetuximab combined with chemoradiotherapy did not show any beneficial effects compared to chemoradiotherapy alone (31). Riaz, *N et al* have compared concurrent cisplatin-RT-treatment to treatment with cetuximab and RT and found superior outcomes for chemoradiotherapy over treatment with cetuximab and RT (32). Side-effects of cetuximab are mainly dermatological (rash, acne) but fever, nausea, and vomiting also occur. Side-effects occur more frequently in combination with RT, most commonly the acneiform rash. Interestingly, the severity of the rash correlates inversely to improved survival, suggesting it as a prognostic marker of favorable outcome (30).

1.2.4.2 Dasatinib

Dasatinib is a tyrosine kinase inhibitor that targets c-Src, a member of the Src-family (= nonreceptor tyrosine kinases). Activation of c-Src mediates transformation, proliferation, invasion, and metastasis in cancer cells. It is overexpressed in HNSCC, and inhibition by dasatinib induced cell cycle arrest and apoptosis *in vitro*, as well as inhibited migration and invasion (33). Dasatinib is FDA-approved for chronic myeloid leukemia but not yet for HNSCC. A phase II clinical trial has not shown benefit of single treatment with dasatinib in advanced HNSCC (34), but there are data suggesting that the combination of cetuximab and dasatinib may improve the therapeutic effect (35). Moreover, mesenchymal cancer cells have shown greater sensitivity towards dasatinib which opens up for combination treatment possibilities in tumors with EMT-associated resistance (36).

1.3 Influences on treatment sensitivity

Hypoxia, epithelial-to-mesenchymal transition (EMT), and cancer stem cells (CSC) are three known factors to influence treatment sensitivity and it has become evident that they are interconnected. Hypoxia has been shown to trigger induction of EMT (37) and to generate CSC

by hypoxia-induced transcription factors (38, 39). Moreover, it has been shown that epithelial cells can gain stem cell properties during EMT (40), facilitating tumorigenic potential and self-renewal (37). The relation between these three factors is of outmost importance, as they all contribute to treatment resistance. For this reason, a combined approach of several targeting modalities will probably be necessary to improve outcome for the patient.

1.3.1 Hypoxia

Hypoxia (=reduced oxygen availability) is a well-established cause for resistance to radiotherapy in solid tumors (41). It arises when an imbalance between oxygen (O₂) supply and consumption occurs, for instance because of insufficient angiogenesis during rapid tumor growth or dysfunctional and aberrant blood vessels in the tumor. In normal cells, hypoxia usually induces apoptosis and leads to cell death. However, cancer cells can adapt to hypoxic conditions and thereby evade cell death. Oxygen is needed in order to form free oxygen radicals to induce DNA damage and subsequently cell death during radiotherapy (42, 43). Without sufficient oxygen levels the effect of radiotherapy is reduced, and the cancer cells in the hypoxic areas survive and continue to grow. A key mediator in the adaption to hypoxia is the transcription factor HIF-1 (hypoxia-inducible factor-1). HIF-1 activates a number of genes involved in angiogenesis, glucose metabolism, cell proliferation and immortalization, processes tightly linked with malignant progression and cancer cell survival. Hypoxic areas (= pO₂ ≤ 2,5mm Hg) occur frequently in solid tumors (41) and can be determined non-invasively using endogenous biomarkers for hypoxia, e.g. HIF-1 α or CAIX. An association between hypoxia/ HIF-1 α overexpression and treatment resistance/ aggressive tumor phenotype has been observed in HNSCC as well as in many other cancers. (42, 43)

1.3.2 Epithelial-to-mesenchymal transition

EMT is a biological process in which primary epithelial cells lose their characteristic phenotype and function and become either partly or fully mesenchymal (44). During this process they gain

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mesenchymal functions like the ability to invade and migrate through extracellular matrix (45). This process is physiological during embryonic development and in tissue repair processes. However, it also plays a key role in malignant progression of epithelial cancer by providing means for invasion and metastasis (46). The exact mechanism of how the EMT process is induced is not yet clear but one suggestion is that EMT is initiated upon signaling from surrounding tumor stroma cells (44). Next, the epithelial tumor cells lose their polarization and adhesion to the basal membrane and to adjacent cells. A change in morphology occurs from an epithelial phenotype (cobblestone-like monolayered cell) to a more mesenchymal phenotype (spindle shaped cell), and mesenchymal functions are gained. After the change to a more EMT-phenotype the tumor cells can enter the blood circulation and travel to distant sites in the body where they may colonize and form micro- and macro-metastasis. At the end of this process the cells may return to their epithelial phenotype by the inverse process, MET (mesenchymal-to-epithelial transition), and thereby resemble the primary tumor they came from (44). Tumors with EMT features have been linked to a more malignant cancer phenotype, in regards to invasion and metastasis, and worse patient outcome. The phenotype of cancer cells can be determined by expression of markers (e.g. transmembrane proteins, various transcription factors). One of the better characterized features of EMT is the loss of epithelial markers (e.g. decreased E-Cadherin expression) and the increase of mesenchymal markers (e.g. increased N-Cadherin) (37). The loss of E-cadherin is considered a hallmark for EMT and is used as a marker for EMT in head and neck cancer (47). Activation of transcription factors such as Snail or Slug, which activate mesenchymal markers and represses epithelial markers, can also be observed and plays an important role for EMT induction (37). EMT has been associated with chemo-radio-resistance in several cancers including HNSCC (48, 49). The induction of EMT activates genes involved in inhibition of apoptosis and enhancement of cell survival, resulting in cell death evasion and thereby treatment resistance (37). Examples of protein markers that have

been associated with treatment resistance and poor prognosis are Twist, Vimentin, Fibronectin, Snail, Slug, FOX1, N-cadherin among others (50-55).

1.3.3 Cancer stem cells

The theory of CSC in solid tumors is based on tumor heterogeneity, meaning that a tumor consists of subpopulations of cells with divergent phenotypes and with different proliferative potentials (56). Cancer stem cells are a subpopulation of tumor cells that are undifferentiated, have the ability to self-renew and to induce new tumor growth (= tumorigenic cells). The existence of CSC in solid tumors has been suggested to be a cause for treatment resistance and relapses. An explanation is that the current treatments kill a majority of cells and lead to shrinking of the tumor, but might fail to kill CSCs that remain and initiate regrowth of the tumor (56). CSCs in solid tumors may be identified by detection of cell surface markers. This has been successfully done in breast cancer (56) where high expression of the cell surface protein CD44 and low or no expression of CD24 was observed in a small group of tumorigenic cancer cells. In HNSCC, Prince et al showed in cells from primary HNSCC samples that CD44-high but not CD44-low cells gave rise to tumors in mice (57). Furthermore, in HNSCC cell lines, populations with higher expressions of CD44 and CD133 have been identified and these populations have demonstrated a high tumorigenic potential when inoculated in mice (58).

1.4 Personalized medicine

The term personalized medicine has evolved out of the observation that there are inter patient differences in treatment response. In a population of patients with the same diagnosis and the same treatment, some are treated very successfully, some have no treatment effect at all, and others actually get significantly worse during the course of the given treatment (e.g. tumor growth, side-effects). Increased understanding of tumor biology has revealed underlying genetic and molecular differences between tumors of the same histologic type. In personalized medicine we aim to find markers that help us to distinguish patients who will benefit from a

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planned treatment and patients who might be harmed, and subsequently tailor the treatment to the individual patient. Treatment advances have been made in all treatment modalities in HNSCC, as described above. Modern imaging and surgical tools make it possible for the surgeon to excise tumors in a more refined way and even excise those tumors that earlier would have been classified as inoperable. In radiotherapy, new fractioning schedules and volume measuring have made it possible to aim different doses to defined target volumes, so that the side-effects can be minimized and normal tissue better spared. Even new treatment modalities like molecular targeted cancer drugs have arisen and are used in patients with locally advanced cancer. Still, the success rate measured in survival and recurrences is unsatisfactory, and the differences in treatment-response of patients with the same tumor classification constitute a big problem. Personalized medicine might be the solution. However, in order to achieve personalized medicine, predictive biomarkers of treatment response are needed.

1.5 Biomarkers

Biomarkers are biological factors that can be measured accurately and reproducibly, and are related to biological and pathogenic processes and/or to response to treatment. In cancer, biomarkers can be prognostic, providing insight into likely outcome for the patient, independent of treatment, or predictive, helping us to predict response to a specific treatment (59). The identification of prognostic and predictive biomarkers is crucial in order to develop individually tailored treatment plans and may potentially lead to the development of new therapeutic strategies.

1.5.1 How to find biomarkers for cancer

Cancer arises from one single cell and develops progressively in multiple steps over a long time. Chronic exposure to carcinogens, molecular and genetic alterations (DNA structure, tumor suppressor genes or oncogenes) and evasion of various defense mechanisms build the grounds for cancer transformation. Through molecular mapping of cancer, alterations in genes

important in processes such as cell cycle regulation, cell death pathways, and DNA repair have been identified. Hanahan and Weinberg have defined in their „Hallmarks of cancer“ (46, 60), a list of capabilities that a cell has to obtain in order to undergo transformation to cancer (Figure 2).

Figure 2. Modified from “Hallmarks of Cancer” (60). Categories as denoted by Hanahan and Weinberg: blue: acquired hallmarks of cancer; yellow: emerging hallmarks; purple: importance of the tumor microenvironment, green: enabling factors.

When in search for cancer biomarkers it makes sense to investigate factors involved in these processes. In this thesis we have focused on a number of processes and factors involved in the “hallmarks of cancer” and investigated their potential as cancer biomarkers (Figure 3).

Figure 3. Processes and factors investigated in this thesis in relation to the hallmarks of cancer.

1.5.2 Specific biomarkers

1.5.2.1 Survivin

Survivin is the smallest member of the Inhibitor of apoptosis protein (IAP) family. It is commonly expressed in embryonic and fetal tissue, can sometimes be found in proliferating adult tissue, but is rarely detected in terminally differentiated normal tissue. However, overexpression of survivin is well documented in the most common human malignancies (61) and is correlated to tumor progression and treatment resistance (62). Several studies have investigated survivin's role in HNSCC, with contradicting results (63). Survivin was shown to correlate with a more aggressive phenotype and worse survival in SCC from different head and neck subsites (64-66). On the other hand there are studies that have shown a favorable outcome associated with high survivin expression in oral SCC (67, 68). New therapeutic strategies targeting survivin have been developed and have reached phase II trials (69, 70).

1.5.2.1.1 Survivin biology

Survivin has both anti-apoptotic and pro-mitotic functions, related to its subcellular localization and regulated by posttranslational modification mechanisms like phosphorylation and acetylation (Figure 4).

Figure 4.. The functions of survivin shown in relation to its subcellular localization, A) in the cytoplasm, B) in the nucleus. Adapted from (62) and modified.

Predominantly located in the cytoplasm and mitochondria, survivin inhibits cell death mechanisms. First, it can block apoptosis either by indirect binding of caspase-9 via XIAP (X-linked inhibitor of apoptosis protein) or by direct binding to caspases 3/7 (70). Phosphorylation of survivin at Thr34 also blocks apoptosis (62, 70). Binding and thereby inhibiting the proapoptotic protein SMAC/DIABLO (Secondary Mitochondria- derived Activator of Caspase) results in inhibition of autophagy. By this means, cytoplasmic survivin acts cytoprotectively. However, the complete mechanism of inhibition of apoptosis by survivin is not fully known (69). Due to its low molecular weight survivin can enter the nucleus by passive diffusion (71) where it together with Aurora B (a mitotic kinase), Borealin, and INCENP (inner centromere

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protein) forms the mitotic key regulator protein CPC (chromosomal passenger complex) (72). Through this pathway survivin may affect the regulation of mitotic spindle formation and cytokinesis, representing its pro-mitotic function and involvement in cell progression. Ser20-phosphorylation of survivin in the cytoplasm enhances binding to Aurora B kinase, contributing to the formation of the CPC and thereby also acting pro-mitotically. In contrast, Ser20-phosphorylation of survivin attenuates its anti-apoptotic activity by interfering with the binding of survivin to XIAP (62). This illustrates the significance of posttranslational modifications in survivin functions. However, the nuclear export of survivin is not passive but actively regulated by binding survivin via its NES (nuclear export signal) to a nuclear export receptor, the Crm1 (chromosome region maintenance-1) (72). Acetylation of nuclear survivin leads to decreased affinity to Crm1, resulting in nuclear accumulation of survivin, followed by decreased presence in the cytoplasm and increased apoptotic activity (62). Additionally, acetylated survivin also interacts with the oncogene STAT3 (signal transducer and activator of transcription 3), suppressing its oncogenic activity. Thus, nuclear acetylated survivin seems to inhibit cell survival. On the other hand, nuclear survivin has also been shown to play a role in DNA damage repair by enhancing repair capacity (70). The existence of different splicing variants may also play a role in survivin function. Wild type survivin, deltaEx3 (loss of exon 3) and 2b (inclusion of exon 2b) are the most researched in association with cancer (69). However, their contribution to tumor progress, tumor behavior, and treatment response is not completely understood and needs to be investigated further (69).

1.5.2.2 WRAP53

WRAP53 (WD40 encoding RNA Antisense to p53) is a relatively recently discovered gene which has been linked to the pathogenesis of diseases like spinal muscular atrophy (SMA) (73) and dyskeratosis congenita (74), as well as to multiple forms of cancer. Increased levels of the protein WRAP53 β have been demonstrated in cancer cell lines as compared to normal cells (75), and overexpression of WRAP53 β has been linked to poor outcome in patients with HNSCC (75) and in patients with rectal cancer (76).

1.5.2.2.1 WRAP53 biology

WRAP53 is a natural antisense transcript to p53, meaning that, located on the opposite DNA strand, it overlaps p53 in a head-to-head fashion and is transcribed in the opposite direction in relation to p53. Due to variant start exons (α , β , γ), three alternative transcripts of WRAP53 with divergent functions exist: WRAP53 α , WRAP53 β , and WRAP53 γ (function for the latter is yet unknown) (Figure 5).

WRAP53 α acts as a regulator for the tumor suppressor gene p53, both on the mRNA and the protein level, enhancing its induction and stabilization upon DNA damage. The exact mechanism as to how it regulates p53 remains yet unclear (77).

WRAP53 β (also called WDR79 or TCAB1) is not involved in p53 regulation. Nonetheless, it has emerged as a significant factor in tumorigenesis due to its crucial role in processes like telomere elongation, Cajal body maintenance, and DNA damage repair (78).

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Figure 5. A) The transcription of WRAP53 gene in an antisense fashion to p53 and the gene products of WRAP53. B) The functions of WRAP53α and β (78). C) The role of WRAP53β in Cajal bodies.

In this study we focused only on this WRAP53 variant. Due to its WD-40 domain, WRAP53β acts as a scaffold protein. The WD40-repeats allow WRAP53β to facilitate interaction with multiple proteins simultaneously, making it possible for WRAP53β to execute multiple functions independently of each other (78). One main function is the maintenance and stabilization of Cajal bodies. Cajal bodies are dynamic nuclear structures that enhance essential biological processes in the cell by assembling the necessary factors in the same space. This includes processes like ribonucleoprotein (RNP) maturation, spliceosome formation, and telomere elongation. WRAP53β is crucial for the structural integrity of Cajal bodies. It guides the SMN (survival of motor neuron) protein and coilin to the Cajal bodies and stabilizes interactions between them. Downregulation of WRAP53β leads to failed localization of these

proteins and subsequently to the collapse of Cajal bodies (73). Defects in Cajal bodies lead to impaired cell proliferation and splicing rates (73). The SMN protein, a splicing regulatory protein, is involved in the assembly of snRNP (small nuclear ribonucleoprotein) in the cytoplasm. snRNPs compose the spliceosome which catalyzes the splicing process. Cytoplasmic WRAP53 β directs SMN from the cytoplasm to the nucleus by promoting its binding to importin β . In the nucleus, WRAP53 β guides SMN and snRNP to the Cajal bodies, building a scaffold and assuring their function in the splicing process. WRAP53 β also guides scaRNAs (small Cajal body-specific RNA) to the Cajal Bodies (78).

WRAP53 β is also important for telomere elongation and telomerase trafficking (79), one of the hallmarks of cancer (60). Telomeres exist at the end of chromosomal DNA, protecting it. They shorten progressively during the cell's lifetime and when largely eroded induce senescence or crisis, ultimately leading to cell death. Therefore, in order to gain unlimited proliferation cancer cells have to overcome telomere shortening. Telomerase exists rarely in normal somatic cells, but may be highly expressed in cancer cells. TERC (telomerase RNA component) is a member of the scaRNA family. Together with the telomerase reverse transcriptase (TERT), dyskerin and WRAP53 β , TERC constitutes the telomerase holoenzyme that adds telomere repeats to the telomeric DNA, counteracting telomere shortening and thereby cell death (75, 79).

WRAP53 β is also involved in DNA damage response and repair (80). It acts as a scaffold for DNA repair proteins and stabilizes interactions between them at the site of DNA double strand breaks (80). The subcellular localization has been suggested to form the basis for normal function of WRAP53 β (81). It is localized both in the cytoplasm and the nucleus. Little is known about the subcellular trafficking mechanisms of WRAP53 β , but it is suggested that deviant localization may cause dysfunction of WRAP53 β .

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1.5.2.3 HPV and p16^{INK4a}

HPV has emerged as an important risk factor for HNSCC. Overall, there are approximately 179 genotypes, classified in regard to the tissue type that they infect (cutaneous or mucosal), and to the risk for malignant transformation of the host cells (low, medium, high) (19). HPV types 16 and 18, belonging to the mucosal and high-risk subgroup, are relevant in HNSCC. HPV-16 has been shown to play a role primarily in oropharyngeal cancer, whereas HPV-18 is more often found in oral or laryngeal SCC (13). HPV⁺ HNSCCs are nowadays recognized as a different entity of tumors compared to HPV⁻ HNSCCs, regarding both the etiology, tumor behavior, clinical characteristics and prognosis. HPV⁺ HNSCC patients are often younger and lack other risk factors. HPV⁺ HNSCC is often a more aggressive type of cancer with a higher incidence of regional metastasis, but patients in this group have a better prognosis as compared to those with HPV⁻ HNSCC. HPV-16 accounts for approximately 80% of HPV⁺ HNSCCs and is associated with a better prognosis than tumors positive for other HPV-suotypes and HPV-negative tumors (19). The protein p16^{INK4a} (referred to as p16) is used as a surrogate marker for HPV, especially in oropharyngeal SCC, where studies have shown strong correlations between HPV-infection and p16 overexpression (82). Detection of p16 can easily be done by immunohistochemistry. In this thesis the HPV status was investigated by analyzing p16 expression in tumor biopsies obtained before treatment.

1.5.2.3.1 p16 biology

p16 belongs to the INK4 class cell cycle inhibitors. In normal cells it facilitates the binding of pRb (Retinoblastoma protein) to the transcription factor E2F, resulting in cell cycle arrest and subsequent decrease of p16 expression. Without p16, pRb does not bind to E2F and cell cycle progression and mitosis occur, leading to an increase of p16. In this manner p16 expression is regulated by its own feedback mechanism, maintaining the balance between mitosis and cell cycle arrest. In HPV-infected cells however, the HPV DNA encodes for the oncogene E7 that

binds and inactivates pRb, blocking the binding to E2F, resulting in continuous cell cycle progression and overexpression of p16 (Figure 6).

Figure 6. The relationship between high-risk HPV-infection and p16 overexpression.

The oncogene E7 acts differently in low-risk HPV than in high-risk HPV, making p16 overexpression a sensitive marker for high-risk HPV infections (82).

1.5.2.4 EGFR

The epidermal growth factor receptor (EGFR), a member of the ErbB-family, is a transmembrane tyrosine kinase receptor, primarily found in cells with epithelial origin. Upon ligand binding to EGFR, several downstream pathways are activated regulating important cellular processes including cell proliferation, cell survival, apoptosis, cell motility, adhesion, cell differentiation, and angiogenesis (83). EGFR is frequently overexpressed in cancers with

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epithelial origin. In HNSCC, overexpression is found in up to 90% (84) and is associated with a more aggressive tumor phenotype, poorer prognosis, and resistance to treatment (85). EGFR is a target for anti-cancer drugs used in the treatment of advanced HNSCC (see section 1.2.4.1). As described earlier, cetuximab blocks EGFR and hinders downstream activation. EGFR is a monomer in its inactive form. Upon ligand binding it forms an active homodimer (with another EGFR molecule) or heterodimer (with another member of the ErbB-family), thereby activating the intracellular protein-tyrosine-kinase (86). Autophosphorylation of tyrosine residues occurs, eliciting activation of several downstream pathways: 1. JAK/STAT: resulting in cell proliferation and cell survival; 2. PI3K/Akt: resulting in angiogenesis, tumorigenesis, and inhibition of apoptosis; 3. Ras/MAPK/ERK: stimulating cell motility, gene expression, and cell-cycle progression; and 4. PLC γ /PKC: resulting in cell cycle progression, transformation, differentiation and apoptosis (87). There is considerable cross talk between the pathways which may also be activated in an EGFR-independent manner. Thus, it is obvious that resistance to cetuximab may have several different underlying mechanisms. In paper IV we investigated the impact of hypoxia and cetuximab in some of these pathways. It has been postulated that HIF-1 α is an effector molecule in EGFR-signalling pathways, especially the PI3K/Akt pathway. Inhibition of PI3K by cetuximab led to decreased levels of HIF-1 α by blocking HIF-1 α synthesis, resulting in improved intrinsic sensitivity (88).

1.5.2.5 HIF-1 α

HIF-1 is a heterodimeric transcription factor that consists of two subunits: the oxygen dependent HIF-1 α and the nuclear HIF-1 β . Under normoxic conditions HIF-1 α is rapidly marked for degradation whereas it eludes degradation in hypoxic conditions and is translocated into the nucleus where it binds to HIF-1 β and forms the transcription factor HIF-1. HIF-1 binds consequently to hypoxia response elements (HRE), activating genes involved in processes that ultimately lead to adaption to hypoxia (41, 42). HIF-1 α can be detected by

immunohistochemistry and may be used to study hypoxia in tumors. HIF-1 α overexpression is common in many malignancies and correlates to tumor progression and invasiveness (89). Several studies have shown negative correlations of HIF-1 α with outcome and tumor phenotype in HNSCC (reviewed by Swartz et al, (42)). Sasabe *et al* showed an association between increased HIF-1 α expression in OSCC and resistance to radio-chemotherapy as well as a more aggressive tumor phenotype (43). In this thesis we analyzed the association of HIF-1 α expression in tumor cell lines to differences in phenotype and treatment sensitivity between cells cultured under normoxic and hypoxic conditions (paper IV).

1.5.2.6 p53

TP53 is a tumor suppressor gene mutated in more than 50% of human cancers (90). Normally, TP53 encodes for the protein p53 that has a short half-life in normal cells. However, upon DNA damage (e.g. radiation-induced) p53 is stabilized and induces cell-cycle arrest to permit DNA repair to take place, or induces apoptosis in cells that cannot be repaired (91). However, mutated TP53 can encode for a dysfunctional protein that lacks tumor suppressive activity, leading to inadequate DNA-repair and apoptosis evasion. In HNSCC the presence of TP53 mutation was shown to be correlated with decreased overall survival (92).

1.5.2.7 Single Nucleotide Polymorphisms

The most common genetic variation among people are so-called single nucleotide polymorphisms (SNPs). They are DNA-sequence variations that differ by a single nucleotide pair between individuals of a population, meaning (at least) one nucleotide (allele) has changed on a certain position in the genome sequence. 99.6% of the human genome sequence is identical in all human beings. The remaining 0.4% consists of SNPs that make each and every one unique. Those SNPs can change the phenotype of a person and can also be responsible for development and susceptibility to diseases. To be defined as a SNP the variant allele must exist at a single base pair position within the genomic DNA in at least 1% of the normal population

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(93). SNPs are used in medicine as genetic markers to track genes involved in common diseases such as diabetes, cardiovascular disease, mental illness, and cancer. They are also believed to influence not only the frequency of cancer in a population and the onset of cancer in an individual, but also the response to anti-cancer treatments. In paper I, we investigated the association of the following SNPs with radiotherapy response: XPC Ala499Val, XRCC3 Thr241Met, XPD Lys751Gln, XRCC1 Arg399Gln and FGFR4 Gly388Arg, MDM2 SNP 309, p53 Arg72Pro. In paper II, two SNPs in the WRAP gene (rs2287499 and rs2287498), linked to susceptibility to cancer in previous studies (94, 95), were investigated in the setting of HNSCC.

1.5.2.7.1 SNPs in DNA repair genes XPC, XPD, XRCC1, and XRCC3

Previous studies have shown a relationship between SNPs in DNA repair genes and outcome of radiotherapy (96). *Xeroderma pigmentosum complementation group C and D (XPC and XPD)* are proteins involved in the identification of DNA damage and initiation of repair. Both the hetero- and homozygous variant genotypes of the XPC Ala499Val SNP were previously found to be significantly associated with less radiation-induced DNA damage than the wild type (97). The *X-ray repair cross-complementing group 1 and 3 (XRCC1 and XRCC3)* proteins also play important roles in DNA repair and preclinical studies have shown a significant association between the XRCC3 Thr241Met polymorphism and radiotherapy response (98).

1.5.2.7.2 FGFR4 Gly388Arg

FGFR4 belongs to the receptor tyrosine kinase family and ligand binding leads to cell growth, mitosis, and differentiation. About 50% of the population seem to have the FGFR4 Gly388Arg polymorphism (99) that has been linked to aggressive tumor progression and metastasis, and is associated with poor prognosis in HNSCC (100, 101).

1.5.2.7.3 MDM2 SNP309 and p53 Arg72Pro

MDM2 (murine double minute 2) is an oncogene and functions as a negative regulator of p53. Overexpression of MDM2 inhibits p53 function, consequently damaged cells can escape cell

cycle checkpoints and become carcinogenic (102). Polymorphisms in the MDM2 gene can alter the balance between MDM2 and p53 and thereby lower the apoptotic response of the cells and make them more resistant to treatments that depend on apoptosis for cell death. The MDM2 SNP 309 has been associated with poor therapeutic response and poor outcome in HNSCC patients (103). The same results were found for p53 SNP p53 Arg72Pro (103).

2 AIMS OF THE THESIS

The general aim of this thesis was to explore potential predictive and/ or prognostic biomarkers on the protein and gene level in tumor biopsies and cell cultures of HNSCC. Further, we aimed to investigate the role of hypoxia in treatment sensitivity.

The specific aims of the studies were as follows:

- I. To examine a series of proteins and SNPs in HNSCC tumor biopsies in order to investigate their potential value as predictive markers of radiotherapy response.
- II. More specifically, to investigate WRAP53 β and two SNPs of the WRAP53 gene in HNSCC tumor biopsies and to further examine WRAP53 β , survivin, and p16^{INK4a} in a larger study cohort of glottic lSCC.
- III. To investigate the impact of hypoxia and the role of HIF-1 α on treatment response, EMT profile, and the expression of CSC markers in HNSCC cell lines.

3 MATERIAL AND METHODS

3.1 Tumor material

3.1.1 HNSCC biopsies

Tumor biopsies used in papers I, II, and III were obtained from the established tumor collection at the Department of Otorhinolaryngology, Head and Neck Surgery, at the University Hospital of Linköping, Sweden (No 416, The National Board of Health and Welfare in Sweden; approved by the Ethical Committee of Linköping). The tumor collection currently consists of more than 300 tumor biopsies from patients with HNSCC, with ongoing collection since December 2003. After written consent from the patient, an ENT Head and Neck surgeon takes a tissue sample from the tumor in conjunction with diagnostic biopsy procedures at the ENT clinic which means that tumor samples are obtained prior to any treatment. The fresh biopsies are transported to the laboratory where they are registered and processed. One part of the biopsy is immediately frozen in liquid nitrogen and thereafter stored in -70° for later use (e.g. DNA analysis). Other parts of the biopsy are used to establish cell lines and/or are formalin-fixed and paraffin-embedded depending on the size of the biopsy.

3.1.2 HNSCC cell lines

In study I, II, and IV we used HNSCC cell lines established from biopsies in the Linköping tumor collection. Those cell lines have the prefix LK. In study IV we also used HNSCC cell lines from Turku University, Finland, provided by Professor Reidar Grenman. Those cell lines are named with the prefix UT-SCC.

Established LK cell lines were cultured in Keratinocyte-serum free medium (SFM) supplemented with penicillin, streptomycin, and 10% fetal bovine serum (FBS). Fresh medium was given twice a week, and the cells were subcultured at confluence once weekly. Periodical screening for mycoplasma contamination was performed. The UT-SCC cell lines were cultured

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in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with glutamine, penicillin, streptomycin and FBS. In paper IV cells were cultured in hypoxia (1% O₂) to investigate the effect of hypoxia on treatment sensitivity.

3.1.3 Patient data

All data related to the patients and their cancer was collected from the patients' medical charts from the department of Otorhinolaryngology, Head and Neck Surgery at the University Hospital of Linköping, transferred to the registry of the tumor collection, and linked to the obtained tumor sample. The registry is updated regularly and includes patient characteristics (age, gender, date of diagnosis, smoking habits etc.), tumor characteristics (tumor site, TNM-classification, histological grade etc.) and information about treatment and follow-up. Data for tumor biopsies from Helsinki and Karolinska were also linked to similar registers and obtained thence.

3.1.4 Papers I and II

A retrospective cohort of tumor biopsies were gathered from the Linköping tumor collection, including patients diagnosed with HNSCC from 2003 to 2009. The inclusion criterion was treatment with radiotherapy, either as a single treatment or combined with surgery. According to the treatment response, patients were grouped into Non-responders or Responders. The treatment response was determined clinically by an experienced ENT Head and Neck surgeon and the information was obtained retrospectively from the medical charts. A Non-responder was defined by either having a growing tumor during radiation, a remaining residual tumor after radiation, or relapse within 6 months of radiotherapy. A Responder was defined by tumor size reduction during radiotherapy and no sign of relapse within one year after irradiation. Twenty individuals were identified for the Non-responder group whereafter twenty Responders were selected, matched according to tumor localization and histological grade as closely as possible.

The cell lines LK0412 and LK0949 were used for siRNA transfection. Both cell lines are derived from fresh tongue tumor samples. Cells in passages 12-18 were used.

3.1.5 Paper III

149 tumor biopsies of patients with glottic laryngeal squamous cell carcinoma (glSCC) without metastasis classified as T2-T3N0 and with radiotherapy or radiochemotherapy as primary treatment during 1999-2009 were gathered from the archives of Linköping University Hospital (n=10), University Hospital of Helsinki (n=64), and Karolinska University Hospital (n=75).

3.1.6 Paper IV

The cell lines LK0412 (tongue), LK0827 (tongue), LK0923 (larynx), UT-SCC-2 (floor of mouth) and UT-SCC-14 (tongue) were used. The cell lines were selected according to their phenotype (epithelial: LK0412, UT-SCC-2, UT-SCC-14; or mesenchymal: LK0827, LK0923) and their known intrinsic sensitivity for radiation, cisplatin, and cetuximab. Passages from 10-25 were used.

3.2 Immunohistochemistry

Immunohistochemistry (IHC) detects specific cellular components (e.g. proteins) in a tissue sample by visualizing an antigen-antibody reaction. After pretreatment of a formalin-fixed, paraffin-embedded tissue sample with a buffer (to unmask antigen epitopes), endogenous peroxidase is blocked and thereafter a solution with a primary antibody targeting the desired antigen (e.g. the specific protein) is added which binds specifically to the antigen under investigation. After a wash step, a secondary antibody targeting the primary antibody is added. The secondary antibody is labeled with a tracer enzyme, e.g. a horseradish peroxidase enzyme that transforms chromogen into a brown-colored precipitate that accumulates at the site of reaction, thereby visualizing the localization of where the first antibody is bound, and consequently where the targeted antigen is located. Counterstaining with hematoxylin is

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performed for morphologic evaluation. The chromogenic stains can then easily be observed in a light-microscope, the staining pattern revealing information about the protein expression and localization of the investigated protein.

Immunohistochemistry is commonly used to identify specific biomarkers, both in clinical routine and in cancer research. It is a comparably cheap method and widely used in diagnostics in the pathology clinic. Antibodies are commercially available and come with a procedure protocol to facilitate uniform and reproducible analysis. Automated immunohistochemistry also increases the reproducibility and reliability.

3.2.1 Scoring system for IHC analysis

For established biomarkers there are validated scoring systems, e.g. the Hercep Test for HER2-protein expression (104). However, many experimental biomarkers lack validated scoring systems resulting in multiple studies with divergent scoring methods. The most common way of assessing expression of biomarkers by IHC is by scoring both the percentage of positively stained cells in relation to the total number of tumor cells (quantitative/semi-quantitative assessment of positively stained cells) and the staining intensity (qualitative assessment) (105). In the semi-quantitative assessment, the percentage of positively stained cells is usually expressed using an interval system (e.g. 1-10%, 11-50% and so on) and then categorized in groups from e.g. 1-3. The interval systems are defined for each investigated biomarker. In the qualitative assessment the staining intensity is commonly scored from 0 - 3 corresponding to negative, weak positive, moderate positive and strong positive staining. In this thesis the protein expressions were scored by three different investigators independently, blinded to patient data and tumor characteristics, treatment, and outcome. The investigators included one pathologist that confirmed tumor content prior to scoring and two additional researchers (clinicians). In a separate session the scoring results were compared by the investigators and a definitive consensus score was determined by mutual agreement.

3.2.1.1 Papers I and II

For survivin, WRAP53 β , and p53 the percentage of positively stained cells was scored as 0% = 0, <10% = 1, 10-50% = 2 or >50% = 3; the staining intensity as 0 = none, 1 = weak, 2 = moderate or 3 = strong. For WRAP53 β the subcellular localization was scored as nuclear if the staining pattern was predominantly nuclear or equal between nucleus and cytoplasm; and non-nuclear, if there was no staining at all or cytoplasmic staining only. For EGFR membrane-staining was evaluated using the HercepTestTM scoring system (DakoCytomation), which takes into consideration the percentage of positively stained cells and intensity (scores ranging from 0-3, negative to strongly positive). Pictures of IHC staining for survivin, p53, EGFR are shown in Figure 2, paper I and for WRAP53 β in Figure 1, paper II. For p16, all slides were scored by a pathologist for intensity of staining as follows: 0 (none), 1 (weak), 2 (moderate) or 3 (strong). Scores of 2 or 3 were considered positive when more than 70% of the target cells were stained diffusely in both the nucleus and cytoplasm.

3.2.1.2 Paper III

The scoring method for WRAP53 β and survivin used in paper I and II were also used in paper III but with slight adjustments. For staining intensity the scores 2 and 3 were considered as positive, whereas 0 and 1 were considered as negative.

The scoring method of survivin was changed in order to be able to create an immunoreactive score (IRS) as applied by Engels et al (68). The percentage of positive cells was categorized in four groups instead of three [0% = 0, 1-10% = 1, 11-50% = 2, 51-80% = 3 and >80% = 4] and scoring was performed for nuclear and cytoplasmic staining independently.

For the determination of the predominant subcellular localization the difference in staining intensity between nucleus and cytoplasm was used in WRAP53 β , scored in the same way as in paper II. The predominant localization of survivin was categorized into predominantly nuclear, equal nuclear and cytoplasmic or predominantly cytoplasmic.

3.3 Western Blot

Western blot is a semi-quantitative method to separate and identify a specific protein in a mixture of proteins extracted from a cell. Separation is based on the protein's molecular weight and performed by gel electrophoresis, exploiting the fact that proteins of different sizes move with different speeds, and hence distances, through a polyacrylamide gel. The separated proteins are thereafter transferred to a nitrocellulose membrane which is incubated with a primary antibody for detection of the specific protein. For visualization, a secondary antibody labelled with a horseradish peroxidase (HRP) and a chemiluminescent substrate are added. This reaction creates a light-signal that corresponds to the position and amount of the specific protein in the sample (the stronger the signal, the higher the amount of protein). This signal is then recorded using a digital camera. Equal loading is verified by reprobing the membranes with a HRP-conjugated anti-actin antibody.

3.4 Genotyping by PCR-restriction fragment length polymorphism (RFLP)

PCR-RFLP is a technique used to find variations in homologous DNA sequences. We used it to identify specific SNPs in DNA from the tumor biopsies. DNA-fragments containing the SNPs of interest were amplified by PCR. Restriction enzymes were then used to cut the DNA at the restriction enzyme recognition sites, resulting in restriction fragments of various lengths (=restriction digest). By gel electrophoresis the fragments were separated according to their lengths. Visualization was achieved by staining with ethidium bromide. The resulting various staining patterns are characteristic to specific SNPs.

3.5 Gene silencing by siRNA transfection

Small interference RNAs (siRNA, also known as silencing RNA) are short double-stranded RNAs that are used for silencing a particular target gene. After transfection into the cell, siRNAs

become part of a RISC-complex (RNA-induced silencing complex) and are unwound into two single stranded RNAs. One single strand RNA is contained in the RISC complex. It is complementary to the target mRNA and thus recognizes it, binds it and induces its degradation and thereby non-translation of the target gene. Transfection of cell lines with siRNA was used in papers I, II and IV to downregulate the expression of survivin, WRAP53 β , and HIF-1 α in order to investigate the role of these proteins in intrinsic treatment sensitivity and/ or proliferation. Knockdown of 70-80% was achieved in all experiments and verified at mRNA level by quantitative real-time PCR (RT-qPCR), and at protein level by Western Blot.

3.6 Reverse Transcriptase- quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR combines two methods for detecting gene expression in a cell. Reverse transcriptase generates complementary DNA (cDNA) from extracted RNA, reflecting RNA expression of a particular gene in a sample. The cDNA is thereafter amplified by real-time PCR (=qPCR) in an automated system where the amplification of the cDNA is quantitatively measured with the help of a fluorescent dye in real time (in contrast to traditional PCR, where the amplified DNA is first measured at the end of the process). In this thesis we used a 7500 Fast Real Time PCR system for RT-qPCR in order to verify downregulation of specific mRNAs quantitatively. The comparative C_t method was applied to determine expression levels relative to untreated controls. Amplification of two housekeeping genes, GAPDH and β -actin, were used as internal standards.

3.7 Intrinsic treatment sensitivity

Intrinsic treatment sensitivity in cell lines is determined by the fraction of surviving cells/colonies after a given treatment compared to the amount of cells/colonies in an untreated control group. Intrinsic radiosensitivity (IR) was assessed in papers I, II and IV. Cells were

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irradiated with 4 MeV photons generated by a linear accelerator (Clinac 4/100, Varian, Palo Alto, CA, USA), delivering a dose-rate of 2.0 Gy/minute (2, 4, 6, 8, and 10 Gy). Intrinsic sensitivity for cisplatin (ICS), cetuximab (ICmabS), and dasatinib was assessed in paper IV. Increasing doses of cetuximab (15, 30, and 60 nM; Erbitux®, Merck KGaA, Darmstadt, Germany), cisplatin (0.1, 0.25, 0.5 µg/mL) or dasatinib (5, 10, 20 nM; Sprycel®, Bristol-Myers Squibb, New York, USA) were added to the cell cultures and incubated 9 days before evaluation. We used two different methods for the assessment of intrinsic treatment sensitivity: crystal violet assay (papers I, II and IV) and clonogenic assay (papers I and II). All experiments were carried out three times, independently from each other and the means and standard variations were calculated.

3.7.1 Crystal violet assay

The crystal violet assay is a quantative method where the amount of adherent cells is quantified. Cells were seeded into 12-well plates (Corning, Corning, NY, USA) with varying density (1000-3000 cells/cm²) according to the plating efficiency of each cell line. In papers I and II the cells were incubated in 37°C for 48 hours to allow for cell adhesion to the plate surface and then irradiated with 8 or 10 Gy. After another three days, attached cells were fixated with 4% paraformaldehyd for 20 minutes, followed by staining with crystal violet (0.04% in 1% ethanol) for 20 minutes at room temperature. After solubilization in 1% SDS (sodium dodecyl sulphate) the optical density (OD) at 550 nm was measured in a Victor plate reader (EG & G Wallac, Uppsala Väsby, Sweden). To determine the intrinsic sensitivity the average OD₅₅₀ for the untreated control was set to 100%. Subsequently, the percentage of the surviving cells after treatment was determined by comparing the average OD₅₅₀ of the treated cells with the OD₅₅₀ values of the untreated control. In paper IV, 24 hours after seeding the cell lines in 12-well plates, half of the cultures were moved to hypoxic conditions and cultured in 1% O₂. The rest of the cultures were cultured under standard conditions (20% O₂). After another 24h, cetuximab,

cisplatin, or dasatinib was added or cells were irradiated with 2, 4, or 6 Gy. The crystal violet assay was performed 9 days after treatment as described above.

3.7.2 Clonogenic assay

The clonogenic assay (also called colony forming assay) is based on the ability of a single cell to proliferate indefinitely and grow into a large colony (consisting of >32 cells). It assesses the capacity of cells to reproduce and form colonies (or clones, therefore the name „clonogenic“). By comparing the colony-forming ability of treated cells with untreated controls intrinsic treatment sensitivity can be determined. The clonogenic assay is the method of choice to determine the effect of irradiation of cells *in vitro*, but can also be used for chemical compounds. We used this method in papers I and II to confirm our crystal violet assay results. Eight days after irradiation of the cells (0, 2, 4, or 6 Gy) they were fixed in 4% paraformaldehyde and stained with crystal violet. Colonies containing at least 32 cells each were counted on 6 separate plates for each dose. Surviving fraction was determined based on the plating efficiency of non-irradiated control cells.

3.8 Statistics

For survival analysis the Kaplan-Meier method with the endpoints overall survival (OS), disease-free survival (DFS), relapse-free survival (RFS), and disease-specific survival (DSS) was used. Differences between the groups were determined by Log-rank (Mantel-Cox) test. Binary logistic regression was used in papers I and II to determine the association between protein expression in the tumor tissues and treatment response. For analysis of tumor cell lines one-way Anova, followed by Bonferroni post hoc tests were used. P-values ≤ 0.05 were considered statistically significant in all statistical tests used in this thesis. IBM SPSS Statistics version 19.0 was used in paper I, version 21.0 in papers II and IV, and version 22.0 in paper III.

4 RESULTS

4.1 Results paper I

The aim of this study was to examine a series of markers in tumor biopsies obtained from HNSCC patients that were treated with radiotherapy, and investigate possible associations between these markers and outcome of radiotherapy. As mentioned in section Material and Methods, 40 patients that received radiotherapy were included, grouped into Non-responders (n=20) and Responders (n=20) according to treatment outcome. Patient characteristics are listed in Figure 1 in paper I. Smoking habits (not shown in Figure 1) were as follows: 9 non-smokers (6 Non-responders/3 Responders), 18 smokers (8 NR/ 10 R), 5 former smokers (3 NR/ 2R), and 8 with unknown smoking history (3 NR/ 5 R).

4.1.1 Survivin

IHC scoring results for staining intensity of survivin are shown in Figure 7, depicting the distribution in the Non-responder (NR) and the Responder (R) groups.

Figure 7. Survivin expression according to staining intensity in Non-Responders and Responders.

RESULTS

The scoring results of the percentage of positively stained cells was homogenous throughout the study material: 39 of the samples expressed survivin in >50% of the tumor cells, one sample showed no staining at all. Statistically, strong staining intensity of survivin was associated with positive response to radiotherapy (binary logistic regression: $p = 0.003$). Moreover, strong and moderate staining intensity were associated with improved overall survival compared to weak staining intensity (Kaplan-Meier: $p < 0.001$ and $p = 0.009$ respectively, Figure 3, paper I). To evaluate the impact of survivin on radiotherapy response we downregulated survivin by siRNA transfection in two cell lines (LK0412 and LK0949) and studied the effect on radiotherapy response. In both cell lines, significantly increased radioresistance was observed after downregulation of survivin [determined by crystal violet and confirmed by clonogenic assay (shown in Figure 4 in paper I)]. Cells with downregulated survivin also showed attenuated proliferation compared to the negative control.

4.1.2 P53, EGFR, and p16

Percentage of positively stained cells and staining intensity of p53 are shown in Figure 8. No association between p53 expression and radiotherapy response was found. However, in Kaplan-Meier analysis improved overall survival was associated with higher percentage of positively stained cells ($p = 0.015$, Figure 3, paper I). There was no association between staining intensity and overall survival.

Figure 8. The expression of p53 according to staining intensity (A) and positively stained cells (B) in Non-Responders and Responders.

As described in the Material and Methods section another scoring system was used for EGFR, combining the quantitative and qualitative expression of EGFR in one score. The scoring results are shown in Figure 9. No statistically significant associations were found between EGFR score and radiotherapy response or overall survival. Positivity for p16 was found in only two samples (base of tongue and hypopharynx), both in the Responder group.

Figure 9. EGFR expression according to EGFR score in Non-Responders and Responders.

RESULTS

4.1.3 SNPs

All tumor samples were tested for the existence of specific SNPs as described in section 1.5.2.7. The number of tumor samples with a variant allele is listed in Table 1, paper I. No significant associations were found between the existence of a SNP and radiotherapy response. Overall survival was significantly worse for patients with the SNP XPD Lys751Gln compared to those with the wildtype ($p = 0.048$, Kaplan-Meier, Figure 3, paper I). In contrary, patients with the SNP FGFR4 Gln388Arg had a significantly longer overall survival compared to patients with wildtype ($p = 0.010$, Kaplan-Meier, Figure 3, paper I). For the other SNPs tested no significant associations were found with overall survival.

4.2 Results paper II

In this paper, the same study material as in paper I was used to investigate the expression of WRAP53 β in tumor biopsies. Furthermore, we tested 39 of the tumor samples for two SNPs in the WRAP gene (rs2287499 and rs2287498) and analyzed their possible association with radiotherapy response and survival.

4.2.1 WRAP53β

The IHC scoring results of WRAP53β are summarized in Figure 10.

Figure 10. The expression of WRAP53β according to staining intensity (A), positively stained cells (B), and subcellular localization (C) in Non-Responders and Responders.

RESULTS

The subcellular localization was the only variable that differed significantly between the groups ($p = 0.025$, Chi-square-test) and showed significant associations with radiotherapy response ($p = 0.029$, binary logistic regression) and overall survival ($p = 0.014$, Kaplan-Meier, Figure 2, paper II), in which patients with nuclear WRAP53 β showed a favorable outcome. We also examined if a combination of nuclear WRAP53 β and survivin would enhance the significant difference in treatment outcome and overall survival. The combination of nuclear WRAP53 β and strong survivin was associated with the most favorable overall survival compared to other combinations (Kaplan-Meier: $p = 0.001$, compared to non-nuclear WRAP53 β with various survivin expression, Figure 4, paper II). Downregulation of WRAP53 β in the two cell lines [LK0412 (80%) and LK0949(70%)] was achieved using a WRAP53 β -specific siRNA oligonucleotide. Upon downregulation of WRAP53 β , the proliferation rate decreased in one of the cell lines (LK0412; $p = 0.01$) and resistance to radiation increased compared with siRNA-negative controls (crystal violet assay: $p < 0.001$ for both cell lines, Figure 3, paper II). Clonogenic assay confirmed an increased resistance to radiotherapy after silencing of WRAP53 β compared to the negative controls (Figure 3, paper II). The existence of SNPs was demonstrated in 7 tumor samples, in which both investigated SNPs were found consistently together. Frequency of genotypes and the distributions between NR and R are presented in Figure 11.

Figure 11. The frequency of genotypes for SNPs rs2287498 and rs2287499 in Non-Responders and Responders.

Statistically, there were no significant associations between the SNPs and radiotherapy response or SNPs and overall survival, nor an association between the SNPs and the subcellular localization of WRAP53 β .

4.3 Results paper III

The aim of paper III was to further investigate survivin, WRAP53 β , and p16 expression in a larger and more homogenous cohort. One subgroup within HNSCC where predictive markers of radiotherapy response would be extremely helpful is glottic laryngeal SCC classified as T2N0-T3N0. Treatment results in this patient group are suboptimal (106) and one explanation might be the use of organ preservation treatment (RT or CRT) as an alternative to radical surgery (often total laryngectomy). Thus, we gathered 149 tumor samples with glottic laryngeal SCC classified as T2N0-T3N0, primarily oncological treated.

RESULTS

4.3.1 Survivin

148 samples were successfully stained for survivin. One sample was lost due to staining failure. Kaplan-Meier- analysis was performed for each factor itself as well as for immuno-reactive scores determined according to Engels et al (68). Statistically, no significant correlations were found between survival (OS, RSS, DFS, DSS) and survivin expression when analyzing the whole study material. In the subgroup of tumors with predominantly nuclear staining, Kaplan-Meier-analysis showed a similar survival pattern as in paper I with a higher survival rate in patients with strong survivin staining compared to those with weaker staining (58% vs 33%). However, results were not significant ($p = 0.091$, Figure 4, paper III).

4.3.2 WRAP53 β

All 149 samples were successfully stained for WRAP53 β . The tumor samples showed a highly heterogeneous staining pattern within the samples which entailed difficulties in the scoring of the percentage of positive cells. Consequently this variable was excluded from further investigation. Staining intensity and subcellular localization were used to determine the subgroups: nuclear positive samples ($n = 75$, 50%), cytoplasm positive samples ($n = 15$, 10%), and nuclear negative and cytoplasm negative, the latter being combined to negative subgroup ($n = 59$, 40%). Kaplan-Meier-analysis revealed a significant difference in 5-year- DFS between patients with positive nuclear staining compared to patients with positive cytoplasmic staining with a worse outcome for the latter ($p = 0.022$, survival rate: 54% vs 27%, Figure 3, paper III). Moreover, even though the results were not significant, survival rates for patients in the positive cytoplasmic subgroup appeared worse compared to patients whose tumors were classified as negative for WRAP53 β staining (55% vs 27%, $p = 0.072$, Figure 3, paper III).

4.3.3 p16

Eleven tumor samples were classified as p16-positive. There were no significant differences in survival endpoints between p16-negative and p16-positive patients. The exclusion of p16-

positive samples did not impact the significance of the above mentioned results. However, it is noteworthy that patients younger than 60 years showed a higher frequency of p16-positivity compared to those ≥ 60 years ($p = 0.017$). Within this younger age group the following observations were made: non-smokers were more commonly p16-positive ($p = 0.022$) and p16-positive patients developed no recurrences and had a significantly longer DFS compared to p16-negative patients ($p = 0.041$ and $p = 0.021$, respectively).

4.3.4 Additional results

Regarding clinical data, we observed that T3N0 patients primarily treated with chemoradiotherapy had an improved outcome compared to patients treated with radiotherapy only, with regard to 5-year survival endpoints (OS: $p = 0.001$; DSS: $p = 0.018$; DFS: $p = 0.001$; RFS: $p = 0.039$, Table II, paper III) as well as recurrences ($p < 0.001$).

4.4 Results paper IV

The aim of paper IV was to investigate the impact of hypoxia and the role of HIF-1 α regarding treatment response, EMT profile, and expression of CSC markers in HNSCC.

4.4.1 Impact of hypoxia on treatment response

Five cell lines, cultured in normoxic and hypoxic conditions, were treated with cetuximab, cisplatin, dasatinib, and radiation, and differences in treatment responses were determined. Changes in treatment sensitivity were observed mainly in cell lines with a primarily epithelial phenotype (LK0412, UT-SCC-2, UT-SCC-14) as compared to no/minor changes in cell lines with a mesenchymal phenotype (LK0827, LK0923). The most noteworthy observation was that LK0412 cells became distinctly more sensitive to cetuximab in hypoxic conditions which was in contrast to our expectations and to the results from the other cell lines. The results are shown in Figure 1A, in paper IV.

RESULTS

4.4.2 The impact of hypoxia on EMT profile and cancer stem cell markers

As expected, cells cultured in hypoxic conditions went through morphological changes from an epithelial phenotype to a more mesenchymal phenotype. In all analyzed cell lines, cells cultured in hypoxic conditions exhibited looser contact with neighboring cells when compared with cells cultured in normoxia (Figure 12).

Figure 12. Morphological changes in EMT-profile under hypoxic conditions.

Changes in EMT profile were determined by differences in mRNA expression of epithelial and mesenchymal markers, shown in Figure 2A, paper IV. The transformation from epithelial to mesenchymal phenotype was marked by significant downregulation of E-cadherin (UT-SCC-2 and UT-SCC-14) and significant upregulation of N-cadherin (LK0412, UT-SCC-2 and UT-SCC-14) as well as fibronectin and vimentin (for LK0412 and UT-SCC-2, UT-SCC-14 showed a non-significant increase). Moreover, a higher level of the stem cell transcription factors Nanog and Sox1 were shown in cells cultured in hypoxic conditions (Figure 2A and 2B, paper IV).

4.4.3 The impact of HIF-1 α on treatment response, EMT profile, and CSC markers

For further analysis of the importance of HIF-1 α the two cell lines with the most pronounced changes in treatment response in hypoxic conditions were chosen (LK0412 and UT-SCC-14). After downregulation of HIF-1 α by siRNA transfection, normoxic and hypoxic cells were treated with cetuximab and cisplatin. UT-SCC-14 showed only minor changes in treatment response upon downregulation of HIF-1 α . LK0412 cells with depleted HIF-1 α still showed a decreased survival rate upon cetuximab treatment in hypoxia compared to normoxia but to a significantly lower extent than siRNA-negative treated cells. (Figure 3C and D in paper IV). Downregulation of HIF-1 α reversed the above described changes of EMT markers in both cell lines, as reflected by the high expression level of E-cadherin in hypoxia and downregulation of N-Cadherin, vimentin, and fibronectin (Figure 4A in paper IV). In LK0412, Nanog was expressed at a significantly lower level after silencing of HIF-1 α . Otherwise no significant changes in CSC markers were shown.

4.4.4 The impact of hypoxia on EGFR signaling molecules and cetuximab treatment

In order to investigate the impact of hypoxia and HIF-1 α on cetuximab-treatment response, the effects of hypoxia, HIF-1 α depletion and cetuximab treatment on EGFR downstream signalling were analyzed by Western blot (results shown in Figure 5, paper IV). The inhibition of pEGFR by cetuximab in LK0412 cells was significantly greater in hypoxia than in normoxia which is in concordance with the observation that LK0412 cells became more sensitive to cetuximab in hypoxia. The downregulation of HIF-1 α with or without cetuximab treatment did neither attenuate or amplify this effect. In contrast, no changes of pEGFR expression were found for UT-SCC-14 cells. In normoxia, cetuximab treatment led to a reduced activation of pAkt in both cell lines. This effect was not demonstrated in hypoxia but could be induced by HIF-1 α depletion and/ or the combination of HIF-1 α depletion with cetuximab treatment, in which the latter was more effective.

5 DISCUSSION

Today, there are no established, reliable predictive markers for radiotherapy response in HNSCC patients, despite tremendous research efforts. The identification of predictive markers would refine the decision-making process and improve patient outcome by stratifying the patients to an optimal treatment. The studies in paper I, II, and III were initiated to investigate potential biomarkers of radiotherapy response using IHC. Two benefits of using IHC are that the method is reasonably inexpensive and can be easily performed in conjunction with routine clinical diagnostics.

5.1 The impact of survivin on treatment response and survival in HNSCC in general and in glottic ISCC in particular (Papers I and III)

The most interesting finding in paper I was the differential expression of survivin between Non-responders and Responders to radiotherapy. Strong survivin expression was significantly associated with improved radiotherapy response and longer overall survival. These results were in contrast to other studies that reported a poor prognosis in tumors overexpressing survivin (64, 107). In those studies, the expression of survivin was determined by the percentage of positively stained cells only, without consideration of the staining intensity. Moreover, only survival was assessed but not association with treatments. Our results, however, were based on staining intensity only, and we specifically assessed survivin expression in two groups with different treatment responses. Our *in vitro* data showing increased radioresistance in HNSCC cell lines upon survivin-depletion, suggest that survivin may itself play a role in influencing radiosensitivity. We also observed an attenuated proliferation of tumor cells upon silencing survivin, suggesting that survivin's effects on radiosensitivity may be mediated by its effects on proliferation rate. This might explain the seemingly conflicting observations of survivin being linked to a more aggressive tumor growth and yet improving the response to radiotherapy.

DISCUSSION

Decreased proliferation and increased apoptosis upon knock-down of survivin was also shown in cell lines derived from lung cancer and esophageal SCC (108, 109). In agreement with our results, other studies have shown that patients with oral SCC demonstrating high survivin expression have a favorable response to radiotherapy (67, 68). Similar results were shown for patients with breast cancer (110) and colon cancer (111). Those studies revealed a better prognosis and overall survival for patients with nuclear survivin expression.

Though our results for survivin were significant, the small sample size and the heterogeneity of tumor sites are weaknesses in the study, and the results should be interpreted cautiously. Further evaluation of survivin expression was performed in a multicenter study including only patients with T2N0-T3N0 glottic laryngeal SCC (paper III). However, in this study we found no significant association between survivin expression and survival. One possible explanation is variation in the IHC staining procedure in paper I and III, possibly influencing detection of survivin in relation to the subcellular location, as more frequent cytoplasmic survivin staining was noted in the tumor material used for paper III. Interestingly, when we investigated the subgroup of patients with predominant nuclear staining we found a similar survival pattern as in paper I, with a trend towards improved DFS with strong nuclear staining intensity compared to weak nuclear staining intensity. Statistically, this association was not significant and should be interpreted carefully. Another explanation might be differences in treatment modalities (paper I included surgical treatment while paper III only included primary oncological treatment). Moreover, the selection of patients in paper I (NR and R) might have yielded a cohort of patients with extreme outcomes, while the larger study cohort of paper III consisted of patients with more heterogeneous outcomes. Finally, the selection of one specific tumor site (paper III) may contribute to the differing results. The role of survivin in laryngeal SCC might differ from its role in HNSCC from other subsites. There are studies on survivin in laryngeal SCC that have linked survivin expression to an unfavorable outcome (66, 112), and yet others

that, like ours, showed no connection between radiotherapy response and survivin (113). Divergent scoring methods, as well as differences in tumor classification (TNM), and treatment approaches may explain the contradictory results. In conclusion, while our results in paper I suggest a role of survivin in radiotherapy response, no associations between survivin and outcome could be shown in T2N0-T3N0 glottic laryngeal SCC. Survivin's role in other subsites included in paper I might be more relevant than in laryngeal cancer.

5.2 The impact of WRAP53 β on treatment response and survival in

HNSCC in general and in glottic ISCC in particular (Papers II and III)

WRAP53 β has emerged as a promising factor in cancer research and has been suggested as a potential prognostic marker as well as a candidate for targeted anti-cancer therapy (75). Study II in this thesis highlighted the role of nuclear expression of WRAP53 β in radiotherapy response. Nuclear WRAP53 β was more common in Responders to radiotherapy and linked to improved treatment response as well as longer overall survival. These findings were supported by *in vitro* data from two HNSCC cell lines which developed resistance to radiation after knock-down of WRAP53 β . In a study on patients with metastatic rectal cancer, expression of WRAP53 β in metastases correlated to better outcome in patients treated preoperatively with radiotherapy (76), an observation that was not made in non-irradiated patients. These results support our findings that WRAP53 β is of importance for response to radiotherapy. On the contrary, Mahmoudi *et al* showed that high WRAP53 β protein level correlated to poorer outcome and low intrinsic radiosensitivity in HNSCC cell lines, and knock-down of WRAP53 β resulted in increased apoptosis and improved radiosensitivity (75). Our experiments showed lower cell proliferation after depletion of WRAP53 β in one of the cell lines. Since no significant changes in cell proliferation were observed in the second cell line, it seems unlikely that decreased proliferation alone can explain the increased radioresistance in WRAP53 β -deficient cells. Another interesting aspect is the involvement of WRAP53 β in telomere elongation.

DISCUSSION

Telomeres exceeding a certain length correlate to higher radiosensitivity (114). Moreover, telomere dysfunction has been linked to radioresistance in oral cancer cells (115). Depletion of WRAP53 β may lead to impaired telomere elongation and thereby promote radioresistance. These findings support that WRAP53 β is linked to response to radiotherapy and might have a predictive value.

The combination of nuclear WRAP53 β and strong survivin staining intensity was also associated with longer overall survival, however, the combination of the two factors was not significantly better than looking at survivin expression alone (paper II). While logistic regression failed to show an association with radiotherapy response, it is noteworthy that 15 samples in the Responder group expressed the combination of nuclear WRAP53 β and strong survivin versus only three in the Non-responder group. The majority of Non-responders showed non-nuclear WRAP53 β staining and variable survivin expression. A larger study is likely needed in order to better evaluate any additional predictive information provided by the combination of these factors.

Further evaluation of WRAP53 β was performed in the cohort of patients with glottic laryngeal SCC (paper III). In this cohort, positive nuclear WRAP53 β expression was associated with longer disease-free survival, compared to patients with positive cytoplasmic WRAP53 β . Interestingly, there was no significant difference in survival between patients with positive nuclear staining compared to those classified as negative, while patients with positive cytoplasmic staining had a shorter disease-free survival compared to both other groups. This made us question whether the accumulation of WRAP53 β in the cytoplasm may be the relevant prognostic factor in this patient cohort, instead of nuclear WRAP53 β . In agreement with our findings, Kaplan-Meier-curves revealed worse survival for breast cancer patients with negative nuclear/ positive cytoplasmic expression of WRAP53 β (81). The biological relevance of the subcellular localization of WRAP53 β still has to be elucidated. Increased accumulation of

WRAP53 β in the cytoplasm may reflect disrupted intracellular passage into the nucleus, consequently hindering nuclear functions of WRAP53 β . In epithelial ovarian cancer, low nuclear WRAP53 β correlated to poor prognosis. After downregulation of WRAP53 β , impaired DNA repair was observed and a connection between genomic instability and carcinogenesis with low expression of nuclear WRAP53 β was suggested (116). The same consequences may occur if WRAP53 β is trapped in the cytoplasm. Most known functions of WRAP53 β are carried out in the nucleus and little is known about cytoplasmic WRAP53 β functions.

In conclusion, WRAP53 β shows potential as a predictive marker for radiotherapy response, and the most important factor seems to be its intracellular localization. More specifically, it appears that accumulation of WRAP53 β in the cytoplasm may be a negative prognostic factor in HNSCC. Further in-depth analysis is needed in order to elucidate the subcellular functions of WRAP53 β and the significance of its subcellular localization in relation to treatment outcome.

5.3 The role of p16 in papers I - III

The significance of HPV-status in oropharyngeal cancer has led to changes in the TNM-classification that is used by clinicians as help in treatment decision-making. In the American Joint Committee on Cancer Staging Manual, oropharyngeal cancer will now be classified according to high-risk HPV-associated cancer and cancer of other cause (117). It is widely recognized that HPV⁺ oropharyngeal cancer has a favorable prognosis and responds better to radiotherapy. p16^{INK4a} is used as a surrogate marker for HPV in oropharyngeal cancer and is in itself a marker for improved treatment outcome (118). In paper I, only two samples were positive for p16 and both samples belonged to Responders to radiotherapy. Exclusion of these samples did not alter the other findings described in paper I. The probability that cancer in other subsites than the oropharynx is caused by high-risk HPV is at least 5-fold lower and presumably rare (119). Nevertheless, based on limited data, an estimated 7% of laryngeal HNC might be attributed to HPV (119). Interestingly, in paper III, 7% of the tumor samples were positive for

DISCUSSION

p16. The significance of p16 in non-oro-pharyngeal HNSCC is disputed, but there are studies that have shown a prognostic value of p16 in other sites (120). In terms of prognosis, survival outcome did not differ between p16- positive and –negative samples in our study. Nonetheless, it is noteworthy that p16-positivity was more common in patients under 60 years, and was associated with non-smokers, absence of recurrence, and improved DFS. The former two observations have also been described in a study of Kalfert *et al* (121) and Baumann *et al* have described an association between p16-prevalence in laryngeal SCC and younger age (122). Taking these findings together, p16 might have a prognostic value in a distinct subgroup of patients with laryngeal SCC, although further investigations are needed.

5.4 The impact of hypoxia on HNSCC cell lines

Hypoxia, EMT, and CSC are independently acknowledged contributors to treatment resistance in squamous cell carcinomas. In the last years, it has also become clear that these processes are interconnected (123-126), although the exact mechanisms of this interaction are yet unknown. In paper IV our results show that hypoxia induces EMT in three primarily epithelial HNSCC cell lines and renders a majority of the cell lines more resistant to treatment with cetuximab, cisplatin and irradiation. Surprisingly, the LK0412 cell line, also primarily epithelial, became more sensitive to cetuximab-treatment when cultured in hypoxic conditions. In addition, HIF-1 α depletion resulted in increased cetuximab-resistance in this cell line compared to hypoxic cells with retained HIF-1 α . This suggests an involvement of HIF-1 α in sensitization of cells to cetuximab-treatment in this cell line. In agreement with our results, the study of Boeckx *et al* showed hypoxia-mediated sensitization to cetuximab and erlotinib in one HNSCC cell line each (127). However, the role of HIF-1 α in their observations was not tested. As HIF-1 α overexpression has been correlated with poor prognosis and outcome, the combination of drugs targeting HIF-1 α and cetuximab has been suggested in order to gain maximal therapeutic effect (88). Thus, it appears crucial to identify patients whose tumors become more sensitive in the

presence of HIF-1 α , as anti-HIF-1 α therapeutics may actually worsen the treatment response to cetuximab and potentially the patient's outcome. However, this reflection is based on findings in a single HNSCC cell line (one out of five analyzed) and further preclinical studies are needed before conclusions can be made.

We further evaluated possible changes in EGFR downstream molecules driven by hypoxia. In our western blot analysis, we noted that the addition of cetuximab to the cells inactivated pEGFR to a greater extent in hypoxia than in normoxia in LK0412, an effect that was not observed in the UT-SCC-14 cell line (which became less sensitive in hypoxia). Since this effect in LK0412 was unchanged by knock-down of HIF-1 α it seems that there are other factors than HIF-1 α involved in this response. Interestingly, HIF-1 α depletion revoked the inactivation of Akt induced by cetuximab in hypoxia, suggesting that the suspected sensitizing effect of HIF-1 α that we have observed might be related to this EGFR downstream molecule. Li *et al* showed that inhibition of PI3K by cetuximab is imperative for downregulation of HIF-1 α synthesis, and that this downregulation is required for maximum effect of cetuximab (88). It would be interesting to see how PI3K is affected in the LK0412 cell line, to see if an explanation for its sensitization to cetuximab can be found.

In one of our earlier studies, sensitivity to cetuximab-treatment in UT-SCC-14 cells was associated with cetuximab-mediated reduction of pEGFR *in vivo* and *in vitro* (128). In the current study, pEGFR expression was similar in both normoxic and hypoxic conditions in this cell line. No major reduction of pEGFR by cetuximab was noted and knock-down of HIF-1 α did not change the pEGFR expression. Nevertheless, UT-SCC-14 became less sensitive to cetuximab in hypoxia and this change was largely unaffected by HIF-1 α depletion, suggesting that there are other mechanisms involved in the decreased cetuximab response than HIF-1 α . However, hypoxia reversed cetuximab-induced inactivation of pAkt, a result that seems to be HIF-1 α -dependent. Considering the PI3K/Akt pathway as pro-survival, this might explain the

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increased resistance in hypoxia. However, a similar pattern was found for LK0412 which becomes more sensitive to cetuximab in hypoxia, emphasizing our assumption that other mechanisms to hypoxia-related resistance to cetuximab have to be considered, e.g. tumor-specific mutations within the K-ras gene (88).

UT-SCC-2 showed high base levels of HIF-1 α and less distinct differences in cetuximab-reponse between normoxic and hypoxic cells compared to the other epithelial cell lines. HIF-1 α stabilization in normoxic cells has recently been described in prostate cancer, mediated by beta-arrestin 1 (ARRB1) (129). In this study, it is suggested that “pseudohypoxia” contributes to adaption of cancer cells leading to a growth advantage. This might also be the case in our cell line and further investigations on HIF-1 α stabilization and its impact on treatment response might be of interest. In the same cell line, cetuximab reduced HIF-1 α expression more distinctly as compared to the other epithelial cell lines in which only minor reduction of HIF-1 α could be observed. Luwor *et al* proposed that mutations within the PTEN gene render cancer cells more resistant to cetuximab-mediated reduction of the HIF-1 α level (130), which could also be of importance in our observations. Cell lines with primarily mesenchymal phenotype (LK0827 and LK0923) showed no effect of cetuximab on HIF-1 α expression and only a few significant differences in treatment response between normoxia and hypoxia were found in these cell lines. This suggests that in tumors with a more EMT phenotype, mechanisms other than hypoxia are responsible for treatment resistance. In contrast to this, we found that the primarily epithelial cell lines (LK0412, UT-SCC-2, and UT-SCC-14) that responded significantly differently to treatment in hypoxia, underwent hypoxia-induced EMT and expressed increased stem-like transcription factors in hypoxic conditions. These observations were only partially reversed by cetuximab or HIF-1 α siRNA. This suggests, that inhibition of HIF-1 α may not be sufficient to overcome hypoxia-induced EMT and stem-like phenotypes, and that factors other than HIF-1 α are of importance in hypoxia-induced changes towards a more malignant phenotype. Decreased

sensitivity to treatment with cetuximab, cisplatin, and radiation in hypoxic UT-SCC-2 and UT-SCC-14 cells has been attributed to gain of a mesenchymal phenotype. These findings are supported by a study of Holz *et al* (131) that also showed decreased radiosensitivity in HNSCC cells with a mesenchymal phenotype, and is in agreement with our previous findings that the mesenchymal marker FN1 is upregulated in radioresistant cells (50).

In conclusion, our study shows that hypoxia has more impact on epithelial phenotype cell lines than mesenchymal phenotype cell lines in regard to treatment response and phenotype changes. HIF-1 α depletion alone does not reverse hypoxia-induced effects, and it is possible that hypoxia and HIF-1 α play a role in sensitizing cancer cells to cetuximab treatment. Thus, targeting HIF-1 α alone might not be the answer to improve response to anti-EGFR treatment in hypoxic tumors and other mechanisms and targets have to be taken into consideration.

6 CLINICAL RELEVANCE

Identification of novel predictive biomarkers is imperative in order to maximize treatment effect and improve patient outcome. A tumor is a complex structure, with intricate intracellular signaling systems within the tumor cells themselves and with considerable cross-talk between tumor cells and cells in the microenvironment. To concentrate on only one factor will probably not be sufficient. More likely, tumor profiling using a combination of multiple characteristics will be needed. This may require an assortment of assessment methods to detect characteristics spanning from the gene to the protein level.

This thesis suggests that the proteins survivin and WRAP53 β have potential as predictive biomarkers for radiotherapy response in HNSCC and might represent a puzzle piece in the overall picture. IHC-staining of these proteins would be relatively easy to implement in clinical routine, and potentially facilitate treatment-making decisions. The continued search for biomarkers may give rise to new targeted treatment strategies. Moreover, combinations of targeted drugs may prove to yield superior effects. Drugs targeting tumor hypoxia are under investigation and are suggested to optimize the effect of cetuximab. It will be important to further investigate if in some tumors hypoxia can increase sensitivity to cetuximab-treatment and, in that case, to find reliable markers to stratify patients to single treatment with cetuximab or to a combination of targeted drugs.

7 CONCLUSIONS

In summary, the following conclusions can be made from the studies included in this thesis:

- The association of survivin with improved radiotherapy response and overall survival in paper I suggests that survivin may play a role in radiotherapy response.
- In glottic ISCC, however, there were no significant associations between survivin expression and survival, and thus, a predictive/ prognostic value of survivin in this HNSCC subtype could not be demonstrated. Together with the results of paper I, this may suggest that survivin's potential as a biomarker might vary between subsites of HNSCC.
- The subcellular localization of WRAP53 β is associated with radiotherapy response and survival in HNSCC. WRAP53 β appears to be a promising candidate biomarker in HNSCC in general and in glottic ISCC in particular.
- The prevalence of p16^{INK4a}-positive glottic ISCC tumor samples was higher in patients younger than 60 and associated with absence of recurrence and improved DFS, supporting p16^{INK4a} as a possible prognostic marker in this subgroup of patients.
- Hypoxia influences intrinsic treatment response in epithelial phenotype HNSCC cell lines but affects the cell lines to different extents. The sensitizing effect of hypoxia on cetuximab-response as observed in one of these cell lines has to be evaluated further as it suggests that treatments targeting hypoxic areas may not lead to an improved outcome in all patients.
- Hypoxia and HIF-1 α induce EMT and an increase in expression of CSC markers in HNSCC cell lines. Depletion of HIF-1 α does not fully reverse these effects,

CONCLUSIONS

indicating that targeting HIF-1 α might not be sufficient to improve treatment outcome.

8 FUTURE ASPECTS

Determination of biomarker expression by IHC has its pitfalls, including both intra- and interobserver variability (105). A standardized interpretation method is warranted and digital tools such as image analysis may provide a means to more accurate and reproducible analyses. It would be interesting to develop a study where potential HNSCC biomarkers are analyzed using image analysis. In breast cancer, a recent study suggests that digital image analysis is superior to traditional microscopic assessment of the well-established biomarkers estrogen receptor, progesterone receptor, proliferation marker Ki-67 and HER2 (132).

Further investigation of survivin and WRAP53 β in tumors of the oral cavity would be interesting, as a majority of the tumors examined in study I and II of this thesis were derived from this subsite. It would be interesting to see if these two proteins are more strongly associated with radiotherapy response and survival in this patient group.

As for the impact of hypoxia and HIF-1 α , our preliminary results show no association between HIF-1 α expression (determined by IHC) and survival in glottic ISCC treated with radiotherapy. It would be interesting to study other factors related to hypoxia, for instance HIF-2 α , in relation to treatment outcome in HNSCC.

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