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Predictive Markers of Treatment Resistance in Head and Neck Squamous Cell Carcinoma

Fredrik Jerhammar



Linköping University
FACULTY OF HEALTH SCIENCES

Department of Clinical and Experimental Medicine,

Faculty of Health Sciences, Linköping University

SE-581 83 Linköping, Sweden

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To Frida, of course

“This is not the end. It is not even the beginning of the end.
This is, however, the end of the beginning”
Winston Churchill

ABSTRACT

Head and neck cancer is a common malignancy with approximately 600 000 new cases yearly. Disappointingly, the overall survival has not increased over the last decades. The concept of personalized medicine, i.e. to treat every patient with an individually planned treatment regime has gathered increased interest, but requires the establishment of novel biomarkers that can predict treatment response.

The aim of this thesis is to propose novel predictive single markers or combinations of markers of response to radiation, cisplatin and cetuximab. The general methodology is to evaluate common differences of cell lines resistant to radiation, cisplatin or cetuximab compared to sensitive counterparts.

In paper I, we analyzed the expression of 14 proteins involved in growth control and/or apoptosis by western blot and related them to intrinsic radiosensitivity (IR) in nine cell lines. No factor had a significant correlation to IR on its own. A combination of EGFR, survivin, Bak, Smad4, and Hsp70 had the best correlation to IR ($R=0.886$, $p=0.001$). Additionally, we analyzed the presence of p53 mutations in the cell lines. All cell lines had at least one missense, splice site or loss of transcript mutation. To be able to combine protein expression and presence of p53 mutations we created a system designated the number of negative points (NNP). With this system we could extract that expression of EGFR, survivin, and p53 missense or splice site mutations had the best correlation to IR ($R=0.990$, $p<0.001$).

In paper II we conducted a gene expression microarray analysis of three cell lines, where common deregulations in two cisplatin resistant cell lines was compared to a cisplatin sensitive cell line. From a bioinformatic approach of gene ontology and molecular network analysis, we defined a transcriptional profile of 20 genes. Key findings were analyzed in a larger panel of cell lines, where high MMP-7 expression correlated with higher cisplatin resistance.

Paper III compared 4 cell lines with high IR to a radiosensitive equivalent. Using a similar bioinformatic approach as paper II, we established a transcriptional profile of 14 genes. Analysis in a larger panel of cell lines revealed that FN1 expression predicts higher IR.

Paper IV establishes the cetuximab sensitivity of 35 cell lines of which 12 were resistant and five were sensitive to cetuximab. After whole genome gene copy number analysis of five cetuximab resistant and five cetuximab sensitive cell lines, and verification of key findings in a larger cell line panel, the results show that the amplification of the YAP1 gene is coupled to cetuximab resistance.

In summary, this thesis proposes a number of novel markers of resistance to radiation, cisplatin, and cetuximab which could influence treatment choice in the future, following verifications in primary tumor material.

SAMMANFATTNING

Cancer i huvud- och halsregionen innebär förekomst av en tumör i de övre luftvägarna, närmare bestämt munhålan, näshålan, stämbandena samt övre delen av luftstrupen. Nästan alla huvud- och halstumörer uppstår i skivepitelet, den yttersta delen av slemhinnorna, och därför behandlar denna avhandling endast skivepitelcancer.

I ett normalt epitel finns det en balans mellan celldöd och celldelning. Dessa processer är reglerade av olika signaleringsmolekyler som bestämmer vad cellen ska göra. Obalans mellan celldöd och celltillväxt kan leda till cancer, som är en sjukdom av okontrollerad cellökning orsakad av en ansamling av genetiska fel.

Tumörer i huvud- och halsregionen behandlas vanligen med kirurgi och/eller strålning i kombination med olika cellgifter. Vissa tumörer svarar bra på behandling, medan andra är opåverkade eller utvecklar resistens, vilket får konsekvensen att bara hälften av patienterna blir friskförklarade.

I denna avhandling undersöks hur behandlingsresistenta tumörceller skiljer sig mot känsliga tumörceller. Faktorer som påverkar strålning och två sorters cellgifter undersöks. Om man kan identifiera faktorer som visar att cellerna är känsliga eller okänsliga för behandling skulle dessa kunna undersökas innan behandling startas. På detta sätt skulle man kunna utesluta behandlingsformer som skulle vara ineffektiva och istället skraddarsy en effektiv behandlingskombination. I denna avhandling föreslås ett antal faktorer som påverkar behandlingssvar på cellnivå. Dessa faktorer behöver verifieras i patientmaterial men kan i förlängningen innebära effektivare behandling mot tumörer i huvud- och halsregionen.

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LIST OF PAPERS

This thesis is based on the following papers, which will be referred to by their roman numerals:

- I. Lovisa Farnebo, **Fredrik Jerhammar**, Linda Vainikka, Reidar Grénman, Lena Norberg-Spaak, and Karin Roberg. Number of Negative Points: A novel method for predicting radiosensitivity in head and neck tumor cell lines. *Oncology Reports* 2008; 20(2) 453-61
- II. Anna Ansell, **Fredrik Jerhammar**, Rebecca Ceder, Roland C. Grafström, Reidar Grénman, and Karin Roberg. Matrix metalloproteinase-7 and -13 expression associate to cisplatin resistance in head and neck cancer cell lines. *Oral Oncology* 2009; 45(10):866-71
- III. **Fredrik Jerhammar**, Rebecca Ceder, Stina Garvin, Reidar Grénman, Roland C. Grafström, and Karin Roberg. Fibronectin 1 is a potential biomarker for radioresistance in head and neck squamous cell carcinoma. *Cancer Biology and Therapy* 2011; 10(12):1244-51
- IV. **Fredrik Jerhammar**, Ann-Charlotte Johansson, Jenny Welander, Agneta Jansson, Peter Söderkvist, and Karin Roberg. YAP1 gene amplification is a marker for cetuximab resistance. *Submitted Molecular Cancer Therapeutics*

ABBREVIATIONS

AKT	V-akt murine thymoma viral oncogene homolog
APOE	Apolipoprotein E
ATM	Ataxia telangiectasia mutated
AUC	Area under curve
Bad	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X _L	B-cell lymphoma X _L
Bid	BH3 interacting domain death agonist
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
COX-2	Cyclooxygenase 2
CTNNB1	Catenin (cadherin-associated protein), beta 1
kDa	kilo Dalton
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
FAK	Focal adhesion kinase
Fas	Fas (TNF receptor superfamily, member 6)
FasL	Fas ligand (TNF superfamily, member 6)
FN1	Fibronectin 1
FOS	FBJ murine osteosarcoma viral oncogene homolog
FOXC2	Forkhead box C2 (MFH-1, mesenchyme forkhead 1)
GO	Gene ontology
GOTM	Gene ontology tree machine
Gy	Gray
HIF1A	Hypoxia inducible factor 1 alpha (HIF-1 α)
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
Hsp 70	Heat shock protein 70
IAP	Inhibitor of apoptosis
ICS	Intrinsic cisplatin sensitivity
IL-6	Interleukin 6

IPA	Ingenuity pathway analysis
IR	Intrinsic radiosensitivity
Ki-67	Antigen identified by monoclonal antibody Ki-67
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	Mitogen activated protein kinase
MDM2	Murine double minute 2
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MYC	V-myc myelocytomatosis viral oncogene homolog
MYCN	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived
NNP	Number of negative points
NOK	Normal oral keratinocytes
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
Oct-4	POU class 5 homeobox 1
PCR	Polymerase chain reaction
PDCD6	Programmed cell death 6
PI3K	Phosphatidylinositol 3 kinase
PLAU	Plasminogen activator, urokinase
PTGER4	Prostaglandin E receptor 4
PUMA	p53 up-regulated modulator of apoptosis
qPCR	Quantitative polymerase chain reaction
Rb	Retinoblastoma 1
RNA	Ribonucleic acid
RNAi	RNA interference
SDC4	Syndecan 4
SDS	Sodium dodecyl sulphate
SERPINE1	Serpin peptidase inhibitor, clade E member 1
SNP	Single nucleotide polymorphism
siRNA	Small interfering ribonucleic acid
SMAD4	SMAD family member 4
SP1	Specificity protein 1 transcription factor
Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SSCA	Single strand conformation polymorphism analysis
STAT	Signal transducer and activator of transcription
TAF4B	TATA box binding protein associated factor 4b RNA polymerase II
TGF- α	Tumor growth factor alpha
TGF- β	Tumor growth factor beta
THBS1	Thrombospondin 1
TIMP3	Tissue inhibitor of metalloproteinases 3
TNM	Tumor-node-metastasis
TRPC6	Transient receptor potential cation channel, subfamily C, member 6
TP53	Tumor protein p53

TPPP	Tubulin polymerization promoting protein
UT-SCC	University of Turku squamous cell carcinoma
VEGF	Vascular endothelial growth factor
VLDLR	Very low density lipoprotein receptor
XRCC	X-ray repair complementing defective repair in Chinese hamster cells
YAP1	Yes associated protein 1

INTRODUCTION

CANCER AND THE CENTRAL DOGMA OF MOLECULAR BIOLOGY

The central dogma of molecular biology explains the information flow in cells (Crick 1970). The blueprint of life is stored in the DNA sequence, which is transcribed to messenger RNA and subsequently translated into a protein effector. Cellular defects in this process, such as mutations, gene copy number variation, gene expression alterations or deregulated protein expressions are all implied in cancer, a disease of accumulated genetic faults. Increased knowledge of cancer promoting pathways is important for diagnosis, for understanding how cancer cells circumvent treatment, and for the design of novel drugs.

This thesis addresses the processes that enable cancer cells to survive anti-cancer treatment. The importance of different mutations, gene copy numbers, expression of mRNA and protein levels are taken into account herein, as predictive markers of therapy response are sought. The establishment of reliable

biomarkers of therapy response is vital in order to take the concept of personalized treatment into clinical practice.

CANCER PROMOTING PATHWAYS

Tumor cells obtain several biological capabilities during neoplastic transformation. Examples of such processes are resisting cell death, sustaining proliferative signaling, and activating invasion and metastasis (Hanahan and Weinberg 2011). Many key players of these routes might also affect treatment response. Cancer promoting pathways assessed in this thesis are explained below.

THE P53 PATHWAY

The p53 protein, encoded by the TP53 gene, has been labeled as the guardian of the genome (Lane 1992), implying its imperative role in deciding cellular fate after DNA damage response. Normal function of p53 after DNA damage includes the directing of the cell towards cell cycle arrest or apoptosis (Vousden 2006), which is regulated by its transcription factor activity. After mild damage, cells can arrest and repair, whereas severe damage should cause apoptosis. However, p53 contributes to a number of other processes as well, such as senescence and autophagy (Vousden and Prives 2009).

Cell cycle arrest after DNA damage is characterized by activation of kinases that phosphorylate p53. p53 induces expression of p21 which in turn inhibits cyclin binding to cyclin dependent kinases, resulting in cell cycle obstruction (Leemans, Braakhuis et al. 2011). The induction of apoptosis by p53 is based on the regulation of other genes with p53-responsive elements, such as Bax, Noxa, and PUMA (Zinkel, Gross et al. 2006). The p53 regulated cellular fate is

a complex signaling network that depends on the amount and modification of p53, along with the cofactors present at the p53 targeted promoter (Vousden and Prives 2009).

The TP53 gene is the most frequently mutated gene in head and neck squamous cell carcinoma (Stransky, Egloff et al. 2011). These mutations are predominantly present in the DNA-binding domain, and leads to a loss of function of p53. Loss of p53 also has implications for cellular reprogramming from differentiated to pluripotent stem cells (Menendez, Camus et al. 2010). Knocking down p53 in normal keratinocytes upregulates Oct-4 and leads to increased developmental plasticity (Li, Jin et al. 2011). This further underlines the importance of a loss of p53 function; it drives cells with a diminished control of genomic integrity towards a phenotype of self renewal.

In addition, p53 mutations can generate a gain of function with tumorigenic properties. For instance, mutations in codons 175 and 273, both of which are present in the DNA-binding domain of p53, promotes invasion and loss of directionality of migration (Muller, Caswell et al. 2009).

The main negative regulator of p53, MDM2, is expressed with aid of p53 transcription factor activity, and normally directs p53 to degradation. Since the DNA-domain mutations of p53 perturb its DNA-binding, MDM2 is expressed to a lesser extent in these cells. As a consequence, mutant p53 often accumulates in cells that harbor mutations.

HUMAN PAPILLOMA VIRUS (HPV)

Apart from the abundant p53 mutations in HNSCC, HPV infection is also tightly connected to this pathway. The E6 and E7 genes are encoded in the HPV genome and have great oncogenic potential, affecting cell cycle progression in two major ways. The E6 protein inhibits p53, which in turn disturbs negative

regulation of cell cycle progression (Leemans, Braakhuis et al. 2011). E7 binds the Rb proteins, which enables E2F transcription factors to induce S-phase entry (Tribius, Ihloff et al. 2011). These molecular implications of HPV infection drive proliferation, and ultimately malignant transformation.

APOPTOSIS

Apoptosis is a process of programmed cell death, which is frequently deregulated in cancer. Evading cell death counteracts the deletion of abnormal cells that are genetically unstable or otherwise damaged. Since DNA damage is the direct cytotoxic effect of many types of cancer therapeutics, deregulated apoptosis is a proficient way of escaping drug induced cell death (Fulda 2009).

Cellular homeostasis is balanced by a number of proapoptotic and antiapoptotic factors. When cellular damage or insult results in exceeding proapoptotic signaling, a program of cellular degradation starts (Fulda 2009). Cells disintegrate into smaller apoptotic bodies, which are removed by macrophages. Importantly, apoptotic cell death is characterized by maintained membrane integrity, keeping the inflammatory response to a minimum.

Initiation of apoptosis occurs from two distinctive but interconnected routes, the extrinsic and the intrinsic pathways. The extrinsic pathway is initiated from ligand binding to a cell membrane bound death receptor. The Fas death receptor and its ligand Fas ligand (FasL) exemplify such signaling, which upon binding is followed by activation of caspases that are the effectors of apoptotic cell death. FasL is predominately expressed on the surface of activated T-cells, but can also be present in a soluble form (Sproll, Ballo et al. 2009). Expression of FasL has been detected in HNSCC (Das, Khare et al. 2011). The intrinsic pathway of apoptosis initiation is characterized by mitochondrial membrane permeability which leads to cytochrome c release. Cytosolic cytochrome c forms the apoptosome which leads to caspase activation and apoptosis.

Overexpression of antiapoptotic factors, such as Bcl-2, Bcl-X_L, and survivin, are reported in various cancer types and leads to decreased cellular response to damage (Li, Boehm et al. 2007; Altieri 2008). Similarly, downregulation of proapoptotic factors such as Bax, Bad, Bak, and PUMA impairs the cell death program.

EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR)

The epidermal growth factor receptor is a 170 kDa transmembranous protein which upon ligand binding dimerizes, autophosphorylates and communicates signaling resulting in gene transcription of proliferation-, migration-, and survival-associated genes. EGFR ligands include amphiregulin, epidermal growth factor (EGF), tumor growth factor alpha (TGF α), and epiregulin. The downstream signaling cascade of EGFR is complex, involving five major pathways; the mitogen-activated protein kinase (MAPK), phospholipase C, phosphatidylinositol 3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT), signal transducer and activator of transcription (STAT), and Src/Focal adhesion kinase (FAK) pathways (Laurent-Puig, Lievre et al. 2009).

EGFR is frequently overexpressed and gene amplified in HNSCC (Ratushny, Astsaturov et al. 2009; Szabo, Nelhubel et al. 2011), exemplifying the sustained proliferative signaling hallmark of cancer (Hanahan and Weinberg 2011). Advanced tumors (stages II-IV) show a higher expression and gene amplification of EGFR than tumors in stage I (Ryott, Wangsa et al. 2009). Shin et al. concludes that EGFR upregulation happens in two steps, the moderate up-regulation of EGFR expression in normal epithelium adjacent to tumor and the further up-regulation of EGFR in the change from dysplasia to squamous cell carcinoma (Shin, Ro et al. 1994).

EPITHELIAL MESENCHYMAL TRANSITION (EMT)

The potential of cells to progress from epithelial to a more mesenchymal state is important during many developmental processes. EMT is also implicated in carcinogenesis, and characterized by loss of epithelial differentiation and a movement towards invasiveness and malignancy (Lee, Dedhar et al. 2006). Prominent features that divide epithelial and mesenchymal architecture are the cell-cell and cell-matrix contacts. While adhesion in epithelia are mostly E-cadherin mediated cell-cell interactions, the mesenchymal cells rely on integrin mediated cell-matrix adhesion (Guarino, Rubino et al. 2007).

Phenotypical markers of EMT cells are elongated cell shape, increased migration, and invasion. Molecularly, EMT is illustrated by decreased expression of E-cadherin and cytokeratins, increased expression of N-cadherin, vimentin and fibronectin, orchestrated through the transcription factors snail, slug, twist and FOXC2 (Lee, Dedhar et al. 2006).

CELL MIGRATION AND INVASION

Apart from an abnormal cell growth, carcinogenesis is also characterized by tumor cell migration and invasion. Local spreading and metastasizing potential is largely dependent on the matrix metalloproteinases (MMP), which remodel the extracellular matrix. Under normal conditions, the MMPs are strictly regulated by the tissue inhibitors of metalloproteinases (TIMP). A disturbed balance of MMP and TIMP is coupled to various pathological conditions. In tumors, the imbalance represents an amplified MMP activity, leading to increased metastatic potential (Verstappen and Von den Hoff 2006).

Several MMPs are overexpressed in HNSCC, including MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-13 (Rosenthal and Matrisian 2006).

MMPs also have a prominent role in regulating EMT. Additional substrates for MMPs, apart from ECM constituents, are cellular surface molecules such as E-cadherin and CD44 (Kessenbrock, Plaks et al. 2010). MMP-2, MMP-7 and MMP-9 contribute to EMT in different carcinomas (Orlichenko and Radisky 2008).

HEAD AND NECK CANCER

Head and neck cancer is the sixth most common cancer worldwide with approximately 600 000 patients diagnosed yearly (Parkin, Bray et al. 2005). For 2009, there were 998 reported new HNSCC cases in Sweden, corresponding to approximately 2% of all Swedish cancer cases (Cancer incidence in Sweden 2009, the national board of health and welfare). The survival numbers of this malignancy is still low, only 40-50% live five years after diagnosis (Leemans, Braakhuis et al. 2011).

RISK FACTORS

HNSCC arise sporadically, and a number of life style related risk factors that influence disease progression have been identified. Tobacco smoking and alcohol have a additive effect, and are connected to 75% of all HNSCC (Argiris, Karamouzis et al. 2008). Swedish moist snuff is not related to head and neck cancer development (Luo, Ye et al. 2007; Lee 2011).

HPV infection is another risk factor for developing head and neck cancer, mainly of oropharyngeal origin. Approximately 60% of oropharyngeal cancers are HPV positive (Ang, Harris et al. 2010), corresponding to about 20% of all HNSCC (Leemans, Braakhuis et al. 2011). In Sweden, the proportion of HPV infected oropharyngeal tumors have increased significantly over the last decades

(Nasman, Attner et al. 2009). Patients with HPV-positive tumors, however, have a significantly reduced risk of death (Ang, Harris et al. 2010; Tribius, Ihloff et al. 2011).

CANCER PROGRESSION

Head and neck cancer consist mostly of squamous cell carcinomas which originate from keratinocytes of the mucosal lining of the upper aerodigestive tract, including the oral cavity, pharynx and larynx.

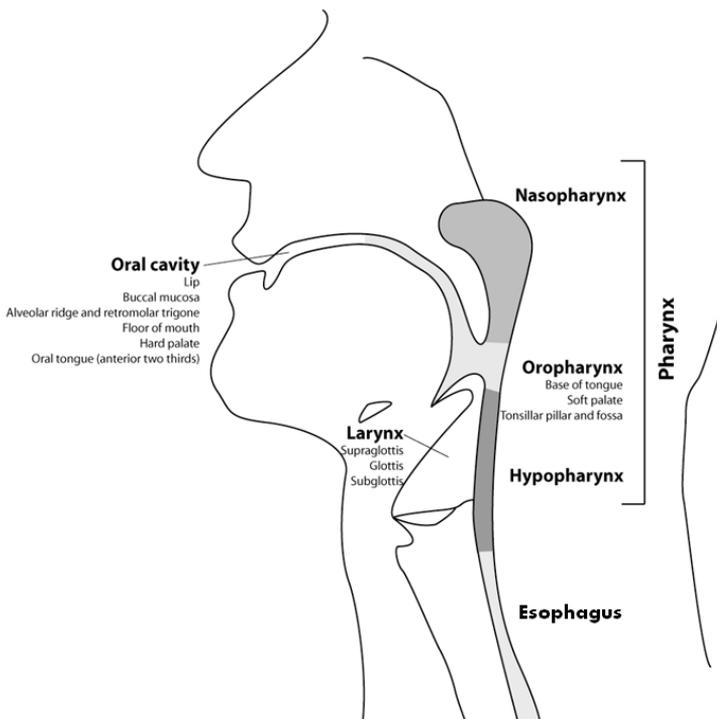


Figure 1. Regions from which head and neck squamous cell carcinomas originate.

Although the epithelia of variable sites of the upper aerodigestive tract are somewhat different, the general structure is similar. The proliferating cells of the epithelium are present in the basal layer. As cells mature they migrate, manifest

altered keratin expression, and ultimately end up lining the mucosa as flattened terminally differentiated cells (Hansson, Bloor et al. 2001; Reibel 2003). Keratinocytes are mostly eliminated after terminal differentiation. Defective differentiation is a route to cancer development, elucidated by the fact that head and neck cancers have been shown to frequently harbor mutations in genes that regulate squamous differentiation (Stransky, Egloff et al. 2011). Genes involved in the loss of oral keratinocyte differentiation have also been associated with poor survival of HNSCC patients (Ceder, Haig et al. 2011).

Dysplasias, such as leukoplakia or erythroplakia, are non cancerous lesions that could potentially undergo malignant transformation. Head and neck cancer patients often present multiple dysplasias or malignant lesions in the upper airway epithelium (Hunter, Parkinson et al. 2005). The process, often referred to as field cancerization, means that a mucosal area consists of cells that harbor genetic or epigenetic changes associated with cancer (Leemans, Braakhuis et al. 2011). After progression of a part of such a field into invasive carcinoma, and subsequent resection of the tumor, the mucosa might still contain genetically altered cells. This is a possible source of local recurrences or of second primary tumors. About 50% of patients with advanced disease will experience local recurrences (Argiris, Karamouzis et al. 2008).

HEAD AND NECK CANCER TREATMENT

The prognosis is good for a patient diagnosed with early stage disease (stage I or II), favorable outcome of up to 90% has been reported (Argiris, Karamouzis et al. 2008). These patients are treated with surgery and/or radiotherapy, depending on the site of the primary tumor. However, two thirds of HNSCC patients are diagnosed with locally advanced disease (stage III and IV), for which the

survival is significantly lower. The treatment options in the advanced stages are surgery, radiotherapy, chemotherapy, or concurrent chemo-radiotherapy.

RADIATION

Radiotherapy remains a core treatment for HNSCC. Efforts are made to enhance the dose delivery to the tumor and to reduce the toxic effects on normal tissue by novel approaches such as intensity modulated radiotherapy (IMRT) and altered fractionation schedules. The IMRT technique renders a possibility to adjust the radiation dose according to the three-dimensional structure of the tumor, which ultimately minimizes the exposure to surrounding normal tissue. Such therapy has been shown equally effective as conventional radiotherapy, but with significantly less late toxicities (Lee, de Arruda et al. 2006).

Studies of altered fractionation schedules, such as accelerated fractionation and hyperfractionation, have been frequently reported over the last decade. Bourhis *et al.* conducted a meta-analysis with the purpose of comparing accelerated fractionation to hyperfractionation in which 15 trials were included. They conclude that altered fractionation schedules improve survival of HNSCC patients, and that hyperfractionation was the best type of altered radiotherapy (Bourhis, Overgaard et al. 2006).

CHEMOTHERAPY

A panel of chemotherapeutic agents including taxanes, anti-metabolites and platinum compounds are utilized in the clinic. The platinum compound *cis*-diamminedichloroplatinum(II), mostly known as cisplatin, is regarded as a standard agent, and is often used in combination with radiation or other compounds. Induction therapy, where often cisplatin is combined with 5-

fluorouracil and sometimes also docetaxel has gathered increased interest (Posner, Hershock et al. 2007).

The standard treatment regimen for unresectable tumors is chemoradiotherapy, because of the higher survival numbers compared to radiation alone (Adelstein, Li et al. 2003). A large meta-analysis by Pignon *et al.* establishes that chemoradiotherapy is slightly superior in terms of absolute survival (8% higher at 5 years from diagnosis), although associated with an increased toxicity (Pignon, Bourhis et al. 2000). Mucositis and other complications are common in concurrent chemoradiotherapy which is widely used but, due to the high toxicity, somewhat debated.

TARGETED THERAPY

Targeted therapy is an interesting and promising field in cancer therapeutics. The drug industry has launched an arsenal of compounds, often targeting growth factor receptors or their downstream effector molecules. Mostly investigated in HNSCC are different agents targeting EGFR. A number of monoclonal antibodies, interfering with extracellular ligand binding, and small molecule tyrosine kinase inhibitors, blocking the intracellular ATP-binding pocket of EGFR have been designed and tested in various solid tumors, with varying success rates (Wheeler, Dunn et al. 2010).

EGFR tyrosine kinase inhibitors such as erlotinib and gefitinib have been approved for other cancers, but have not proven sufficient for consideration as head and neck cancer treatment (Wheeler, Dunn et al. 2010). Gefitinib was recently reported as a non-efficient additional treatment to chemoradiotherapy in a phase II study of 226 HNSCC patients (Gregoire, Hamoir et al. 2011).

One agent that has shown clinical potential in the treatment of HNSCC is Cetuximab (C225, Erbitux®), which is a monoclonal antibody directed towards

the epidermal growth factor receptor (EGFR). Its activity at the ligand binding site of the receptor inhibits the downstream signaling and ultimately hampers the EGFR-coupled gene expression. Clinical studies have implied the usefulness of cetuximab in different combinatory regimes. Bonner *et al.* demonstrated that there is an increase in overall survival after treatment with cetuximab in combination with radiotherapy as compared to radiotherapy alone (49.0 months compared to 29.3 months) (Bonner, Harari et al. 2006). After these promising results, cetuximab was the first targeted therapy to be approved for treatment of HNSCC. It has been shown that Cetuximab could also improve overall survival when combined with platinum and 5-fluorouracil as compared to platinum and 5-fluorouracil alone (10.1 months to 7.4 months) (Vermorken, Mesia et al. 2008).

PREDICTIVE AND PROGNOSTIC MARKERS

A prognostic marker is a biological trait that can be used to estimate the outcome of a particular disease. A predictive marker, on the other hand, is a characteristic which relates to the treatment response. Clinical diagnostic evaluation of cancer progression includes histologic appearance, tumor grading, lymph node involvement and presence of distant metastasis. These are valuable as prognostic indicators, but are of limited importance in prediction of treatment responses (Rodrigo, Ferlito et al. 2005).

Numerous prognostic HNSCC markers are available in the literature, but few have a validated clinical value. As previously described, advanced tumor stage and nodal involvement are indicative of low survival. HPV infection increases the risk of developing HNSCC, but is also a positive factor for favorable outcome (Chang and Califano 2008).

The establishment of prognostic markers is important for the understanding of tumor progression and identification of new therapeutic targets (Haddad and Shin 2008).

PREDICTIVE MARKERS OF TREATMENT RESPONSE

The course of cancer progression is complex and involves multiple deregulated biological pathways. The growing arsenal of novel therapeutic compounds is interesting but in order to reach significantly elevated survival numbers, the establishment of predictive markers of treatment response is crucial.

RADIATION

Proteins that control apoptotic cell death have impact on the cellular response to radiation. The balance between anti- and proapoptotic members of the Bcl-2 family regulates cellular fate, and altered expression has been suggested to influence cellular radioresponse (Polverini and Nor 1999; Guo, Cao et al. 2000; Condon, Ashman et al. 2002). Antiapoptotic proteins (such as Bcl-2 and Bcl-X_L) and proapoptotic markers (e.g. Bad, Bak, Bax, and PUMA) have implied a possible predictive significance in need of further evaluation (Kato, Kawashiri et al. 2008). Inhibitors of apoptosis (IAPs) are also crucial regulators of apoptosis. Survivin is an IAP which is usually unseen in terminally differentiated adult tissue, but is detected in many human cancers (Altieri 2008). Overexpression of survivin has been linked to cellular evasion of apoptosis following irradiation (Roberg, Jonsson et al. 2007). Factors that are involved in radiation induced DNA-damage response have been proposed as potential markers (e.g. ATM, p53, XRCC proteins) but studies have so far been unable to validate their clinical importance (Sarbia, Ott et al. 2007; Silva, Homer et al. 2007). Another

important biological factor which influences the response to radiotherapy is hypoxia. The generation of reactive free radicals that damage DNA is enhanced by the presence of oxygen. Radiation is therefore more effective in patients with well-oxygenated tumors (Silva, Homer et al. 2007), which is also exemplified by decreased overall survival in patients that overexpress the hypoxia marker hypoxia inducible factor 1 α (HIF-1 α) (Aebersold, Burri et al. 2001). Tumor cell proliferation, or rather repopulation between treatment occasions, seems to be another factor that hampers local control after radiotherapy (Silva, Homer et al. 2007). The relative advantages of altered fractionation schedules might be explained by impeded tumor cell repopulation (Bourhis, Overgaard et al. 2006).

CISPLATIN

Cisplatin was shown to have antitumor activities in 1969, as it inhibited the growth of sarcoma and leukemia cell lines injected in mice (Rosenberg, VanCamp et al. 1969). Cisplatin functions through the forming of DNA-adducts, primarily by intrastrand crosslinking, and involves pathways such as DNA repair, cell cycle arrest, and apoptosis, which all converge on p53 signaling (Siddik 2003; Wang and Lippard 2005). Treatment response does not correlate to p53 expression in HNSCC (Sarbia, Ott et al. 2007). However, reduced cisplatin sensitivity of head and neck squamous cell carcinoma correlates with mutations affecting the nuclear localization of p53 (Mandic, Schamberger et al. 2005), indicating an importance for functional p53 in the signaling cascade in response to cisplatin treatment. In concordance with this, it has been shown that Nutlin mediated p53 reactivation restores cisplatin sensitivity (Koster, Timmer-Bosscha et al. 2011).

Apoptosis signaling through Fas-FasL has also been shown to influence cisplatin response in epithelial cancer cells. Lower FasL expression is coupled to cisplatin resistance in ovarian cancer cells (Schneiderman, Kim et al. 1999), and cisplatin

treatment leads to increased expression of Fas and FasL in HNSCC cell lines (Sundelin, Roberg et al. 2007).

TARGETED THERAPY

Studies have shown clinical achievement of cetuximab, and recently several markers of resistance have emerged. EGFR copy number has been related to worse prognosis of HNSCC (Nakata, Uzawa et al. 2011), but was recently shown unrelated to cetuximab response in combination with platinum/5-Fluorouracil (Licitra, Mesia et al. 2011). KRAS mutation is predictive for cetuximab failure in colorectal cancer (Karapetis, Khambata-Ford et al. 2008). However, KRAS mutations are rarely seen in HNSCC (Sheikh Ali, Gunduz et al. 2008; Kasten-Pisula, Saker et al. 2011). Similarly, BRAF mutation is a marker for cetuximab resistance in colorectal cancer (Di Nicolantonio, Martini et al. 2008), but low mutation grade makes it of limited importance in HNSCC (Kondo, Tsukuda et al. 2011). Interestingly, severity of skin rash induced by cetuximab can predict response to the drug, and the patients with grade 2 rash live longer than patients with no or grade 1 rash (Bonner, Harari et al. 2010).

It is not definitive that active signaling through the EGFR is increased when the total EGFR is increased. A recent publication from Thariat et al. in fact shows that the fraction of phosphorylated receptor declines as compared to total EGFR as EGFR expression escalates (Thariat, Etienne-Grimaldi et al. 2012).

Recently, a panel of biomarkers was assessed for prognostic value in cetuximab containing therapy. Serum IL-6 and VEGF were associated with tumor response to cisplatin, docetaxel and cetuximab (Argiris, Lee et al. 2011). To monitor response to therapy is a valuable clinical tool. However, markers that can guide clinicians in treatment planning are not abundant at present.

MICROARRAY ANALYSES IN HNSCC

Microarray technology has opened the possibility of simultaneous analysis of thousands of genes. Tests that predict clinical outcome on the basis of gene expression are likely to positively affect cancer treatment and increase the use of a personalized medicine approach (van't Veer and Bernards 2008). Questions have been raised regarding the reproducibility of microarray results, and there are examples of gene expression signatures that, even though validated in independent patient cohorts, show relative little overlap of genes (Singh and Pfister 2008). It has also been stated that differential gene expression on transcript level matches protein abundance to only about 40% (Tian, Stepaniants et al. 2004). However, deregulation of gene product levels correspond to a higher extent regarding functional categories, albeit not for individual genes (Shankavaram, Reinhold et al. 2007).

The search for predictive markers of treatment response is based on comparison between sensitive and resistant samples, where high throughput analyses promises upcoming accomplishments. However, gene expression studies of head and neck carcinomas have primarily focused on identifying signatures of cancer progression (Chung, Parker et al. 2004; Lee, Yang et al. 2007; Yan, Yang et al. 2007; Ye, Yu et al. 2008), representing interesting aims although distinct from predictive marker evaluation.

Extensive data are emerging in the field of predictive chemotherapeutic markers. Several studies propose gene expression signatures that can guide the use of chemotherapeutic drugs in breast cancer management (Potti, Dressman et al. 2006; Bonnefoi, Potti et al. 2007). Takashima et al. established a gene list of 38 markers differently expressed in esophageal cancer cell lines resistant to cisplatin (Takashima, Ishiguro et al. 2008), involving functional categories like

cell cycle and signal transduction. Radiotherapy related transcriptomic analyses of HNSCC have mainly compared pre- and post-treatment gene-expression profiles (Higo, Uzawa et al. 2005; Ishigami, Uzawa et al. 2007; Amundson, Do et al. 2008), detecting deregulated functional categories such as cell cycle and cellular growth and proliferation (Ishigami, Uzawa et al. 2007). Arguably, basal gene expression patterns observed in the cell lines before treatment is likely to serve better as a predictor of intrinsic treatment resistance (Amundson, Do et al. 2008).

DNA MICROARRAYS

Genomic imbalance and accumulating mutations drive carcinogenesis. In addition to mutations, tumors often present large gene copy number variations. In head and neck cancer, commonly reported variations are gain of 5p, 7p, 8q, 11q, 20p, 20q and loss of 3p, 8p and sex chromosomes (Martin, Reshmi et al. 2008; Ambatipudi, Gerstung et al. 2011; Bhattacharya, Roy et al. 2011; Leemans, Braakhuis et al. 2011). Comparing common genetic variations of a specific phenotypic trait in multiple tumors could provide novel biomarkers of importance for disease progression or treatment response.

AIM

The overall aim of this thesis is to propose predictive biomarkers of resistance to treatment of head and neck squamous cell carcinomas.

More specifically, this thesis aims to:

- Propose a predictive model of intrinsic radiosensitivity by combining markers on protein and genetic level
- Propose biomarkers of intrinsic resistance to cisplatin by bioinformatic processing of transcriptional differences between cisplatin resistant and cisplatin sensitive cell lines
- Propose biomarkers of intrinsic radiosensitivity by bioinformatic processing of transcriptional differences between radiation resistant and radiation sensitive cell lines
- Propose biomarkers of intrinsic cetuximab resistance by genome wide assessment of gene copy number differences between cetuximab resistant and sensitive cell lines

MATERIALS AND METHODS

CELLS AND CULTURE CONDITIONS

Extensively used in the papers of this thesis are the UT-SCC-series of cell lines established by Professor Reidar Grénman, University of Turku, Finland (Grenman, Burk et al. 1989; Pekkola-Heino, Jaakkola et al. 1995). From a total of 35 UT-SCC cell lines we have, for each study, chosen a number of cell lines to represent different parts of the treatment sensitivity scale with respect to intrinsic radiosensitivity, intrinsic cisplatin sensitivity, and intrinsic cetuximab sensitivity. In addition, ten cell lines of the LK-series established by Dr Karin Roberg were used in paper IV.

PAPER I

Nine HNSCC cell lines, representing different parts of the *in vitro* radiosensitivity spectrum, were selected for this study. Normal oral human

keratinocytes (NOK), cultured as previously described (Sundqvist, Liu et al. 1991), where also included in the study.

PAPER II

Three cell lines from the UT-SCC-series were selected on the basis of their intrinsic cisplatin sensitivity (ICS). Two of the cell lines (UT-SCC-2 and UT-SCC-24) were resistant to cisplatin and one was extremely sensitive (UT-SCC-12). The ICS of 39 cell lines was previously established in these cell lines by a clonogenic assay (Farnebo, Jedlinski et al. 2009).

PAPER III

In this study five UTSCC cell lines were selected for microarray analysis, to represent different parts of the *in vitro* radiosensitivity spectrum. Four of the cell lines showed high resistance and one cell line was sensitive.

PAPER IV

The intrinsic cetuximab sensitivity was assessed on 35 UT-SCC cell lines. Five cetuximab resistant cell lines (UT-SCC-1A, UT-SCC-2, UT-SCC-16, UT-SCC-25, and UT-SCC-29) and five cetuximab sensitive cell lines (UT-SCC-15, UT-SCC-17, UT-SCC-23, UT-SCC-46, UT-SCC-47) were assessed on a gene copy number array. Apart from these ten cell lines, an expanded panel consisted of ten cell lines established by Dr Karin Roberg as described elsewhere (Roberg, Ceder et al. 2008). These also had the distribution of five cetuximab resistant (LK-0806, LK-0850, LK-0855, LK-0863, LK-0923) and five cetuximab sensitive (LK-0858, LK-0917, LK-0921, LK-0924, and LK-0942).

INTRINSIC RADIOSENSITIVITY (IR)

The intrinsic radiosensitivity of HNSCC cell lines of the UT-SCC series was previously determined using a 96-well plate clonogenic assay (Grenman, Burk

et al. 1989; Grenman, Carey et al. 1991; Pekkola-Heino, Jaakkola et al. 1995). Survival data as a function of radiation dose were fitted by a linear quadratic equation, and the area under curve (AUC) was obtained by numerical integration (Fertil, Dertinger et al. 1984). For each cell line a minimum of three experiments were performed. The average IR in a large panel of HNSCC cell lines was 1.9 for head and neck cancer of all sites (N=37) (Pekkola-Heino, Jaakkola et al. 1995). The cell lines used in this thesis span from an IR of 1.4 (UT-SCC-9) to an IR of 2.6 (UT-SCC-24).

INTRINSIC CISPLATIN SENSITIVITY (ICS)

The cytotoxic effect of cisplatin was determined in 39 HNSCC cell lines by a clonogenic assay (Farnebo, Jedlinski et al. 2009). Tumor cells were seeded into six-well plates at concentrations of 200-400 cells/cm² depending on the plating efficiency of each cell line. After 24 h cells were exposed to 1µg/ml cisplatin for 1 h and incubated for another nine days before fixation in 4% paraformaldehyde. Cells were then stained with 2% Giemsa and colonies containing 32 cells or more were counted. The number of colonies in untreated cells was set to 100%, and the number of colonies of treated cells was expressed as a percentage of the control value. In each experiment all cell lines were exposed in triplicate and the mean value was used for statistical analysis.

INTRINSIC CETUXIMAB SENSITIVITY

The intrinsic cetuximab sensitivity was determined in 35 UT-SCC cell lines by a crystal violet assay. Cells were seeded into 12-well plates in concentrations of 500-5000 cells/cm² according to the plating efficiency of each cell line. The mean concentration was 1300 cells/cm². After 24 h, cetuximab was added to a final concentration of 30nM and left for 48 h when cells received fresh medium.

Cells were cultured for a total of ten days before fixation in 4% paraformaldehyde and stained with 0.04% crystal violet in 1% ethanol. After washing in water, the crystal violet stain was solubilized in 1% sodium dodecyl sulphate (SDS), and absorbance at 550 nm was measured. A ratio between the absorbance for cetuximab treated cells and its untreated control provided the intrinsic cetuximab sensitivity.

WESTERN BLOT

The western blot technique is used to examine the presence of a protein in a cell lysate. In Paper I, the protein abundance was defined by computer software that measures the optical density of the protein bands and results were correlated to the expression in NOK. We also correlated the bands to β -actin expression to adjust for unequal loading between samples. The resulting values were designated adjusted relative densitometric (ARD) values.

POLYMERASE CHAIN REACTION-SINGLE STRAND CONFORMATION POLYMORPHISM ANALYSIS (PCR-SSCA) AND DNA SEQUENCING

In Paper I we wanted to examine the impact of protein expression and p53 mutations on intrinsic radiosensitivity of cells. Therefore, analysis of the occurrence of p53 mutations was performed on the cell lines. The p53 gene was amplified by PCR, and the cell lines which displayed an altered p53 sequence were subject to further analysis. DNA sequencing was then performed in order to isolate the specific nucleotide sequence.

DNA sequencing was also used in paper IV to establish the status of the KRAS and BRAF genes in ten cell lines.

NUMBER OF NEGATIVE POINTS

In Paper I, a system of comparing protein expressions with gene mutations was established, named Number of Negative Points (NNP). The expression (ARD values) of the fourteen proteins was classified into four groups (0-3 points); no (0-1.50), small (1.51-4.50), intermediate (4.51-7.50) or large changes (7.51-) in expression compared to NOK. All fourteen proteins analyzed were classified in this same point system with the same above levels for the ARD values.

The p53 mutations were arranged into three groups depending on their type. Group one included all mutations, group two contained the splice site and missense mutations and the third group contained loss of transcript. Each p53 mutation received one point in the NNP system.

MICROARRAYS AND BIOINFORMATIC ANALYSIS

In papers II and III of this thesis, we wanted to perform unsupervised analysis to determine patterns of gene expression differences between resistant and sensitive samples. In paper IV, a global assessment of gene copy numbers was used with similar intent. The microarray technology permits simultaneous determination of transcript levels or gene copy numbers of all human genes in a sample by the assessment of up to several million data points.

EXPRESSION MICROARRAYS

The arrays used in Papers II and III (Affymetrix Human Genome U133 Plus 2.0) analyses the expression of 38 500 genes. The vast amount of data that is extracted from these analyses requires appropriate statistical and bioinformatic processing in order to yield useful results. Both papers have a similar setup, although the treatment sensitivity addressed is IR in Paper II and ICS in Paper III. The overall ambition was to use an unsupervised approach to reveal

biomarkers of treatment response. By comparing the differently expressed transcripts that are shared between resistant cell lines compared to a sensitive cell line we isolate the phenotype of resistance to the treatment and examine the pathways that are mutually changed. Subsequent bioinformatic analysis is likely to reveal more information on pathways regulating treatment resistance.

GENE ONTOLOGY TREE MACHINE (GOTM)

The Gene Ontology Consortium (<http://www.geneontology.org>) started out as a collaborative project between three model organism databases (FlyBase, Saccharomyces Genome Database and Mouse Genome Database). The project aims to provide a consistent vocabulary to explain the function of gene products from three main categories, that is, biological process, molecular function and cellular component. We have in Papers II and III compared resistant cell lines to sensitive cell lines and analyzed the differences in gene expression for enrichment of gene ontology categories. To carry out this analysis we used Gene Ontology Tree Machine (GOTM, <http://bioinfo.vanderbilt.edu/gotm>), which is a web based statistical hypergeometric test applied for enrichment analysis of Gene Ontology (GO) categories.

INGENUITY PATHWAY ANALYSIS (IPA)

The IPA knowledge base (<http://www.ingenuity.com>) contains roughly 2 million peer-reviewed articles from the scientific literature and is manually curated by PhD scientists. By using the IPA tool to construct molecular networks we wanted to find key players of the results from the gene ontology analysis. Selection of key regulators in the networks was based on interactions with at least three altered transcripts (Staab, Ceder et al. 2007).

COPY NUMBER MICROARRAYS

In paper IV, the differences in gene copy number was assessed globally to determine differences between cetuximab resistant and cetuximab sensitive samples. The arrays used were Affymetrix SNP 6.0 arrays, and after quality controls in the Genotyping Console software (Affymetrix), the main data analysis was performed in the GeneSpring GX 11.5 software (Agilent) which supports all major commercially available microarray platforms and provides multiple analytical tools.

QUANTITATIVE PCR

In papers II and III, the microarray analysis implied several biomarkers that were differently expressed in the resistant cell lines compared to the sensitive cell lines. Firstly, we wanted to verify the relative expression differences implied from the microarray analysis. Secondly, we wanted to assess these markers in independent tumor material, i.e. a larger panel of cell lines. By quantitative PCR (qPCR) analysis, the relative expression values of mRNAs are detected by the $\Delta\Delta C_t$ method described elsewhere (Leutenegger, Mislin et al. 1999).

In paper IV, qPCR was used for two purposes. Firstly, to verify the relative gene copy number of selected markers implied from the microarray, and secondly, to assess the mRNA expression of selected genes.

RNA INTERFERENCE

RNA interference (RNAi) is performed to downregulate the expression of a specific gene. Small interfering RNAs (siRNA) are short nucleotides that interfere with target mRNA and the gene silencing is achieved by the RNA-

inducing silencing complex (RISC). RNA interference is a good way of hypothesis testing when searching for biomarkers. Downregulation of a predictive marker leading to increased treatment sensitivity is a good indicator for the importance of the marker.

RESULTS AND DISCUSSION

RESULTS PAPER I

A requirement for better outcome in cancer therapy is to discover markers of treatment response. Many markers have been implied in preclinical studies, but few have shown a clinical potential and have failed to make their way into clinical practice (Lothaire, de Azambuja et al. 2006).

This study aimed to combine markers that were previously suggested as markers of response to radiation. The purpose was to correlate these markers to the intrinsic radiosensitivity of nine HNSCC cell lines. The origin and IR of the cell lines are summarized in Table I of paper I. Expression of 14 proteins involved in growth control and/or apoptosis were analyzed along with presence of p53 mutations in the nine cell lines. Using western blot densitometric expression values adjusted to actin expression and standardized to expression in NOK, assessment of the importance of each marker for the IR of the cell lines was made. The analyzed proteins were: Bad, Bak, Bax, Bcl-2, Bcl-X_L, COX2, Cyclin

D1, EGFR, Hsp70, MDM2, p53, PUMA, Smad4, and survivin. None of the proteins could alone show a significant correlation to IR.

EGFR is commonly overexpressed in HNSCC and is highly anticipated as a therapeutic target. The expression of EGFR differed greatly among the cell lines (Figure 1A, paper I), and was higher than NOK in all cell lines except UT-SCC-12A. Survivin was upregulated in eight out of nine cell lines (Figure 1B, paper I). COX2 was upregulated in five out of nine cell lines. Since the aim of this study was to find a combination of markers correlating to IR, the ARD values of these three proteins were combined, which resulted in a significant correlation to IR ($r=0.0825$, $p=0.006$). However, when COX2 was omitted, the correlation was even stronger ($r=0.878$, $p=0.002$).

The expression values of the Bcl-2 family proteins (Bad, Bak, Bax, Bcl-2, Bcl-X_L, and PUMA) are summarized in table III of paper I. Bcl-2 was only upregulated in UT-SCC-34, whereas Bad, Bax, Bcl-X_L, and PUMA were overexpressed in several cell lines. Bad, Bax and PUMA had a negative r-value in the correlation analysis, consistent with their pro-apoptotic function. Therefore, when the ARD values of multiple markers were assessed, proapoptotic markers were subtracted from the total value. Only Bak could positively influence the correlation to IR. When subtracted from EGFR and survivin, Bak slightly increased the correlation to IR (Figure 2A, paper I).

Smad4, Hsp70, and Cyclin D1 were overexpressed in seven, eight, and nine of the cell lines, respectively. Smad4 and Hsp70 but not Cyclin D1 increased the correlation values when added to EGFR, survivin and Bak (Figure 2B, paper I).

The p53 protein was overexpressed in three of the cell lines and its regulator Mdm2 was overexpressed in two (table IV, paper I). They did not increase the correlation to IR together with the other markers. Since p53 is of great importance in tumorigenesis it was additionally analyzed on gene level, for

detection of p53 mutations. All cell lines displayed at least one p53 defect (Table II, paper I), whereas the UT-SCC-77 cell line had three mutations.

Changes in protein expression and mutations of genes are important events in cancer development. These events deregulate cancer promoting pathways and can also influence the response to cancer therapy. We therefore created a system where the influence of deregulated protein expressions and p53 mutations could be simultaneously assessed. This was named number of negative points (NNP). Using a multivariate computer calculation, all possible combinations of factors were analyzed. The combination of factors with the strongest correlation to IR was EGFR, survivin, and splice site or missense p53 mutations ($r=0.990$, $p<0.001$; table V, paper I).

DISCUSSION PAPER I

The possibility of using a multimarker approach analyzing several markers of different cancer promoting pathways is generally affirmed as a way forward for cancer research, including HNSCC (Thomas, Nadiminti et al. 2005). To this end, we wanted to combine a number of proteins involved in cell growth and apoptosis, and to evaluate the importance of these markers in radioresistance. Additionally, the study aimed to establish a method for combining protein expression and gene mutations for correlation to intrinsic radiosensitivity.

None of the markers had a significant correlation to IR on its own. EGFR had the best individual correlation ($R=0.619$, $p=0.075$) and with the addition of survivin, a significant correlation was reached. Combining additional markers, we found that some increased the correlation while others decreased it. Keeping the markers that increased correlation and discarding others, we conclude that in this system the combination of the ARD values of EGFR, survivin, Bak, Smad4, and Hsp70 resulted in the highest correlation to IR. These results support the

concept that a panel of markers need to be assessed when predicting the response to therapy.

EGFR contributed to the signature with the highest correlation to IR. Blockade of EGFR with cetuximab in combination with radiotherapy has been suggested as a successful combinatory regime in HNSCC (Bonner, Harari et al. 2006). Five of the cell lines in this study overexpressed EGFR. Three of these had extensive overexpression. However, it is not definite that a tumor overexpressing EGFR has increased EGFR signaling (Thariat, Etienne-Grimaldi et al. 2012). On the other hand, high EGFR expression was part of a panel of markers (with low Ki-67 and low p53) that predicted low response to radiotherapy in HNSCC (Suwinski, Jaworska et al. 2010). Undoubtedly, EGFR is central in HNSCC development. However, to be able to predict treatment sensitivity, it might be necessary to take other players of the EGFR pathway into account.

Survivin also contributed to the highest correlation to IR signature. Although the mechanisms are not fully elucidated, survivin is important in mitosis and inhibits apoptosis (Mita, Mita et al. 2008), possibly by the inhibition of caspases (Marioni, D'Alessandro et al. 2010). Survivin is upregulated in HNSCC, and has been previously coupled to radioresistance (Roberg, Jonsson et al. 2007), and downregulation of survivin has been shown to radiosensitize cells (Sah, Munshi et al. 2006). Although much evidence points to the tumor promoting effects of survivin, high survivin expression has also been coupled to good prognosis after radiotherapy (Freier, Pungs et al. 2007). The limited expression of survivin in normal tissue makes it a possible therapeutic target, and several small molecules and gene therapeutic strategies are in clinical trials (Altieri 2008). These accumulated results suggest that survivin is an interesting predictive marker for radioresistance in need of further evaluation, and that inhibition of survivin

might be a proficient way to target a tumor, possibly with the addition of radiotherapy.

To be able to take both protein expression and mutations of p53 into account simultaneously, we created the NNP system. The best combination was EGFR, survivin and p53 splice site or missense mutations. A computer based multivariate analysis came to the same conclusion. Imperfect p53 signaling has large implications on cellular DNA damage response, and therefore on radiosensitivity as well. The TP53 gene is mutated in around 60% of HNSCC tumors (Stransky, Egloff et al. 2011), and to an even higher extent in cell lines. In this study, all cell lines had some kind of p53 defect (Table II, paper I). The protein expression of p53 did not increase correlation to IR when combined with other factors. It is not clear if the western blot analysis detected normal or mutated p53, however, since the p53 mutations were very important in predicting IR it seems that in our system, the loss of functional p53 overshadows the tumor promoting effects of mutant p53.

The location of the p53 mutation is also important, where mutations in the DNA binding domains are graver than others. Peltonen et al. showed that tumors that harbor p53 mutations in the DNA-binding regions are more resistant to radiotherapy, and that these patients have poorer survival (Peltonen, Vahakangas et al. 2011). This study also shows that the type of mutation in p53 is important, as splice site and missense mutations seem to affect IR to a higher degree than a loss of transcript. Six cell lines have missense or splice site mutations in p53. Five of those have mutations in the DNA-binding domain (UT-SCC-24A, -77, -2, -33, and -19A). UT-SCC-12 has a mutation in the tetramerization domain.

Suwinski et al. used a similar approach as the NNP method. With the purpose of separating HNSCC patients responding or not responding to postoperative irradiation, they assigned points corresponding to low expression of the proliferation marker Ki-67, low expression of p53, and high EGFR expression,

as assessed by immunohistochemistry. They found that patients with a low score of this multimarker approach did not benefit from treatment, whereas the patients with a high score did (Suwinski, Jaworska et al. 2010).

This study suggests that a combination of several markers is needed when predicting response to radiotherapy. Future analyses should be aimed at verifying these results, using a larger tumor material and a more sensitive quantitative method.

RESULTS PAPER II

Cisplatin is widely used for treatment of HNSCC in combination with surgery and radiotherapy. Tumor resistance to cisplatin leads to persisting disease and fatalities, but patients still experience the side effects. Biomarkers that could predict treatment resistance to cisplatin would hinder unnecessary suffering and guide clinicians to a better treatment choice.

This study aimed at finding predictive markers of cisplatin response. In contrast to paper I, where already proposed markers were assessed, we here performed an unsupervised study which utilized the microarray technology to examine the transcription patterns of three HNSCC cell lines, one sensitive and two resistant to cisplatin treatment.

A way of gaining functional understanding of large microarray data is the use of various systems biology approaches. Bioinformatic processing and categorization of detected changes is needed to make high throughput data concrete. In this study, enrichment of gene ontology categories in the gene ontology tree machine, and network analysis by ingenuity pathway analysis were used. Finally, the importance of key findings was assessed by qPCR in a panel of 25 cell lines where cisplatin sensitivity was previously established (Farnebo, Jedlinski et al. 2009).

The global mRNA expression pattern of the two cisplatin resistant cell lines was compared to the sensitive cell line, and 781 genes were found to be differently expressed in both resistant cell lines.

Using the GOTM-tool, this gene list was found to enrich 11 functional categories, summarized in table 2 of paper II. The biological processes involved were development, cell adhesion, cell differentiation, cell migration, and response to virus. Under molecular function, calcium ion binding and interferon

compared to the sensitive, and therefore selected for qPCR verification. All but TIMP3 verified the microarray results.

APOE, CTNNA1, THBS1, MMP7, and MMP13 were analyzed in a panel of 25 cell lines, in order to evaluate the importance in a representative head and neck cancer material. For each marker, the cell lines were divided into two groups corresponding to high or low expression. The cisplatin resistance was then compared between the groups. This analysis showed that the cisplatin resistance was significantly higher in the group with high expression of MMP7 ($p=0.0013$, Figure 2A, paper II). Furthermore, high expression of MMP13 associated to cisplatin resistance but was not statistically significant ($p=0.058$, Figure 2B, paper II). The expression of APOE, CTNNA1, and THBS1 did not correlate to cisplatin resistance ($p=0.67$, 0.30 , and 0.27 , respectively).

DISCUSSION PAPER II

In this study, we wanted to determine the intrinsic dissimilarities between cells that are resistant or sensitive to cisplatin. This was carried out by comparing the global mRNA expression of two cisplatin resistant cell lines to a sensitive counterpart, in order to obtain a transcriptomic signature that signified cell survival after cisplatin treatment. A list of genes was obtained which was analyzed with GOTM in order to obtain a biological interpretation of deregulated genes. Cell differentiation and development has been previously accompanied with acquired cisplatin resistance in ovarian (Chen, Yan et al. 2007) and HNSCC cells (Negoro, Yamano et al. 2007). Consistently, these biological processes were here found to be involved in intrinsic cisplatin resistance as well (Table 2, paper II). Additional biological processes reported here are cell adhesion, cell migration and response to virus.

The IPA analysis also provides common functions coupled to the molecular networks, which are similar to the gene ontologies given by GOTM analysis. In an analysis of cisplatin resistant cell lines, cellular movement and cell-to-cell signaling and interaction were reported as top functions (Yamano, Uzawa et al. 2009). Although with a relative little overlap of genes in the reported networks, these functions were also top functions in our system (Table 3, paper III). Connective tissue related functions were also reported in both studies. The kind of bioinformatic approach used here is not widely reported in relation to cisplatin and HNSCC; however, functions and gene ontologies related to cellular migration, cell adhesion, and interaction with the extracellular space are recurring.

The network analysis identified 20 key regulators (Table 3, paper II). Eight of these have been previously coupled to regulation of cisplatin response, namely CTNNB1, FN1, FOS, HIF1A, MYC, TGFB1, TIMP3, and TP53 (Nakamura, Kato et al. 2003; Helleman, Jansen et al. 2006; Yang, Wang et al. 2006; Sasabe, Zhou et al. 2007; Cao, Bennett et al. 2008; Fraser, Bai et al. 2008; Mishra, Bisht et al. 2008; Zhang, Qian et al. 2008).

Based on their differential expression in cisplatin resistant and sensitive cell lines, six key regulators (APOE, CTNNB1, MMP-7, MMP-13, THBS1, and TIMP3) were selected for analysis on a larger cell line panel. This analysis showed that MMP-7 expression was able to separate cell lines in two groups with differing sensitivity. Additionally, MMP-13 showed a strong tendency to influence cisplatin response ($p=0.058$).

MMPs are central in carcinogenesis because of their potential to influence cellular migration and angiogenesis. MMP-7 has been shown to facilitate tumor invasion by activating MMP-2 and MMP-9 (Wang, So et al. 2005; Wang, Reierstad et al. 2006). MMP-7 is mainly produced in tumor cells, and expression of MMP-7 is associated with poor survival in HNSCC (Weber, Hengge et al.

2007). MMP-13 is expressed by tumor cells, impacts metastatic spread, and associates to shorter survival in HNSCC (Luukkaa, Vihinen et al. 2006).

Failure of apoptotic pathways is one of the major mechanisms of resistance to platinum containing drugs, such as cisplatin (Wang and Lippard 2005). For example, the restoration of cisplatin sensitivity by reactivation of p53 was dependent on apoptotic signaling via Fas/FasL (Koster, Timmer-Bosscha et al. 2011). One of the substrates of MMP-7 is Fas, which leaves Fas without an extracellular domain, unable to induce apoptosis (Strand, Vollmer et al. 2004). This is a known mechanism of resistance to oxaliplatin (Almendro, Ametller et al. 2009), another platinum containing drug. Interestingly, MMP7 also cleaves FasL, which leads to a disruptive apoptotic signaling (Mitsiades, Yu et al. 2001). Previous work from our lab showed that cisplatin treatment upregulated Fas in UT-SCC-24 cells (Sundelin, Roberg et al. 2007), which was one of the cisplatin resistant cell lines used in paper II. Interestingly, this upregulation did not result in increased apoptosis. The present study proposes that MMP-7 might be responsible for cellular evasion of apoptosis during cisplatin treatment. Future perspectives should focus on the functional impact of these results by blocking or downregulating MMP-7. The already established MMP-7 expression by HNSCC tumor cells proposes a mechanism of chemotherapy failure, which should be tested for predictive value in patient material.

RESULTS PAPER III

The vast majority of HNSCC patient are treated with radiotherapy, most often in different combinatory regimes of surgery or chemotherapy, and sometimes with the addition of cetuximab. Therapy is sometimes impeded by tumor radioresistance, for which there are no biomarkers used in clinical practice today. A set of reliable biomarkers that can aid clinicians in therapeutic choice would potentially have great impact on HNSCC patient survival.

This study aimed to provide markers of radioresistance, by whole genome mRNA expression profiling of five cell lines with differing radioresistance. The radiosensitivity of the cell lines was previously determined by Professor Grénman, and denoted intrinsic radiosensitivity (IR). A high IR number indicates a radioresistant cell line. Comparing four cell lines with high IR to one cell line with low IR, we wanted to extract common expression differences relating to radioresistance. Bioinformatic processing in GOTM and IPA served to elucidate phenotypic characteristics of radioresistance. Key findings were assessed in a panel of 29 cell lines, for verification in a larger tumor material. This panel had an IR average of 2.0, ranging from 1.4 to 2.6.

We found that 552 transcripts shared differential expression between all cell lines with high IR, as compared to the sensitive cell line. By GOTM analysis, we found that a total of 16 GO-categories were enriched (Table 2, Paper III). The biological processes were: cell proliferation, coagulation, death, development, hormone secretion, localization, locomotion, and response to external stimulus. Molecular functions associated with this gene list were: activin inhibitor activity, carbohydrate binding, oxidoreductase activity, profilin binding, receptor activity, structural molecule activity, and specific RNA polymerase II transcription factor activity. Finally, one cellular component category was enriched, i.e. extracellular region.

The genes that enriched the above GO-categories were subject to network analysis using IPA. Results are summarized in Table 3, paper III. This analysis generated eight molecular networks. The top scoring network was associated to cellular movement, growth and proliferation and to cardiovascular development and function, and is showed in Figure 1A of paper III. The genes that interacted with at least three others in the molecular networks were identified, here designated “hub genes”. These were: FN1, PLAU, SDC4, SERPINE1, THBS1, VLDLR, MYCN, TP53, TAF4B, SP1, CEBPA, CEBPB, MYC, and CTNNB1.

The hub genes that were found by microarray to be differentially expressed in the resistant cell lines compared to the sensitive were also examined by qPCR in a panel of 29 HNSCC cell lines. These were FN1, SERPINE1, THBS1, and VLDLR. The cell lines with higher FN1 expression had higher mean IR (1.9 vs. 2.1, $p=0.047$). Consistently, IR correlated well to FN1 expression as tested by a Pearson correlation analysis ($r=0.371$, $p=0.047$). The other markers either failed to verify the microarray expression differences (VLDLR) or did not have different IR in groups with high or low expression. Finally, mRNA expression differences of FN1 implied from the qPCR was verified on protein level by western blot.

DISCUSSION PAPER III

Radiotherapy is the backbone of HNSCC treatment used in combinations with surgery, chemotherapeutic drugs, and targeted compounds. However, tumor radioresistance is a clinical problem. Biomarker detection to exclude radiotherapy for predicted radioresistant tumors would reduce unwanted side effects and increase quality of life for patients. In this study we wanted to compare the global mRNA expression of radioresistant cell lines to a

radiosensitive cell line, in order to find pathways and markers that influence cellular response to radiation.

There were 552 transcripts deregulated in all four radioresistant cell lines compared to the sensitive. With a similar bioinformatic approach as paper II, we firstly applied gene ontology tree machine to the data. Importantly, this is the first assessment of enriched gene ontologies made for HNSCC and radioresistance. However, gene ontology analysis have been performed on HNSCC cells and tissue specimens and have indicated deregulation in cell proliferation, development, signal transduction, structural molecule activity, protein binding and extracellular components (Ginos, Page et al. 2004; Staab, Ceder et al. 2007; Roberg, Ceder et al. 2008; Yan, Chen et al. 2008). Cell proliferation and signal transduction have been reported as important for radioresistance in cervical and lung cancer studies (Kitahara, Katagiri et al. 2002; Guo, Lin et al. 2005).

After IPA analysis, we identified 14 hub genes. Previous connection to HNSCC was found for CEBPA, MYC, SP1, and THBS1 (Bitzer, Stahl et al. 2003; Albo and Tuszynski 2004; Bennett, Hackanson et al. 2007; Staab, Ceder et al. 2007). Four genes, CTNNB1, PLAU, SERPINE1, and TP53 were previously associated to HNSCC and response to radiation. The plasminogen activator PLAU and its inhibitor SERPINE1 are both upregulated in response to irradiation (Fukuda, Sakakura et al. 2004; Schilling, Bayer et al. 2007; Bayer, Schilling et al. 2008). We showed in paper I that mutations in TP53 influences radioresponse, and CTNNB1 has also been coupled to radioresistance mechanisms (Chang, Roh et al. 2008).

When assessed in a larger tumor panel, we here identified that FN1 expression is indicative of radioresistance (Figure 2B, paper III). FN1 is one of the most frequently reported deregulated genes in HNSCC, as identified by meta analysis of studies comparing head and neck cancer to normal mucosa (Lallemant,

Evrard et al. 2009; Lallemand, Evrard et al. 2010). Possible radioresistance from high FN1 expression relates to its interaction with integrins on the epithelial cell surface. Integrins alpha 5 and beta 1 together form the fibronectin receptor, which intracellularly signals via focal adhesion kinase and src to initiate tumor cell motility and invasiveness (Amundson and Smilenov 2011). Intervention of this pathway by a focal adhesion kinase inhibitor radiosensitizes HNSCC cells (Hehlgans, Lange et al. 2009).

FN1 is detectable in saliva from oral SCC patients (Suresh, Vannan et al. 2012), and has been shown to be elevated in 66% of HNSCC patients (Warawdekar, Zingde et al. 2006). A predictive marker that can be identified without an invasive procedure is undoubtedly an appealing possibility.

RESULTS PAPER IV

Cetuximab is a monoclonal antibody directed at the EGF receptor. It is approved for treatment of various solid tumors, e.g. colorectal cancer and advanced stages of HNSCC in combination with radiotherapy. Two genetic markers, KRAS and BRAF mutations, have been found to influence response to cetuximab in colorectal cancer. These mutations are scarcely distributed among HNSCC patients, and alternative markers for resistance are needed. This study aimed at finding novel markers for cetuximab resistance in HNSCC comparing resistant and sensitive cell lines on a whole genome gene copy number array.

Initially, the cetuximab sensitivity was calculated in 35 HNSCC cell lines from a ratio of cetuximab treated cells and untreated controls. The mean cetuximab sensitivity was 0.76, ranging from 0.16 to 1.5 (Figure 1, paper IV). Two cell lines proliferated after cetuximab treatment. Cell lines which had a cetuximab sensitivity of less than 0.5 were considered sensitive, and cell lines exceeding 0.95 were considered resistant. By this definition, twelve cell lines (34%) were resistant and five cell lines (14%) were sensitive.

EGFR is commonly overexpressed in HNSCC, and amplification of EGFR gene copy number is connected with decreased survival (Nakata, Uzawa et al. 2011). We therefore examined if the EGFR status could be related to the cetuximab response. The EGFR protein expression of the cell lines did not correlate to cetuximab sensitivity (Supplementary figure 1A, paper IV). Consistently, EGFR copy number was not different between cetuximab resistant and sensitive cells (Supplementary figure 1B, paper IV).

Five resistant cell lines (UT-SCC-1A, UT-SCC-2, UT-SCC-16, UT-SCC-25, and UT-SCC-29) and five sensitive cell lines (UT-SCC-15, UT-SCC-17, UT-SCC-23, UTSCC-46, and UT-SCC-47) were selected for genome wide copy number analysis. These cell lines were all KRAS and BRAF wildtype. A gene

was considered amplified if the copy number was three or more. To be considered for further analysis, a gene should be amplified in at least four out of five resistant cell lines, and in no more than one sensitive cell line. This analysis detected 39 protein coding genes that were all distributed on two genomic regions, 11q22.1 and 5p13-15 (Table 2, paper IV). Five genes representing the different regions were selected for further studies: TRPC6 (11q22.1), YAP1 (11q22.1), PTGER4 (5p13.1), PDCD6 (5p15.33), and TPPP (5p15.3). No genes were found to be unanimously deleted in the resistant cells and not in the sensitive cells.

For further analysis, ten additional cell lines of which five were resistant and five were sensitive were used along with the ten cell lines assessed on the arrays. To evaluate the importance of the selected genes to cetuximab response, the gene copy number was assessed with qPCR. Only YAP1 amplification showed a tendency to correlate to cetuximab resistance in 20 cell lines (Figure 2A, paper IV). YAP1 mRNA expression was found to be expressed more in cetuximab resistant cells compared to sensitive (Figure 2B, paper IV). This was statistically significant in the ten array cell lines ($p=0.03$), and showed a strong tendency in the expanded cell line panel ($p=0.052$). Not all resistant cell lines have YAP1 amplifications. However, all cell lines that have substantial gene amplifications are cetuximab resistant. In a public gene expression database (<http://www.genesapiens.org>) we found that there is a large variation in YAP1 expression among head and neck cancers (Figure 3, paper IV). Additionally, a subset of head and neck cancers seem to have more YAP1 expression than all other samples in the database.

After YAP1 downregulation UT-SCC-1A showed an increased sensitivity to cetuximab, whereas UT-SCC-25 cells were showed no changes in cetuximab response (Figure 4, paper IV).

DISCUSSION PAPER IV

After the promising results by Bonner et al. in 2006 (Bonner, Harari et al. 2006), cetuximab was the first targeted therapy to be approved for head and neck cancer treatment, to be used in combination with radiotherapy for treatment of advanced stages of HNSCC. However, the use of cetuximab is costly, and since not all patients respond, predictive markers of response are needed. Mutations in the KRAS and BRAF genes have been coupled to clinical resistance in colorectal cancer patients; however, these mutations are not abundant in HNSCC. The present study aimed at providing novel markers of resistance to cetuximab by analyzing gene copy number differences of cetuximab resistant and sensitive cell lines. When analyzing a panel of 35 HNSCC cell lines, we found that the cetuximab response varied greatly. A large group of cell lines were resistant to cetuximab. Interestingly, two cell lines proliferated under cetuximab treatment. If extrapolated to a clinical situation, this shows that selecting the patients that should receive this therapy is important.

The greater part of HNSCC tumors overexpress EGFR, which contributed to the anticipated efficacy of EGFR inhibition. However, neither high EGFR expression nor amplified EGFR genes are sufficient biomarkers for cetuximab response. We found that EGFR protein expression did not correlate to cetuximab response in our system. Consistently, there was no difference in EGFR copy number between cetuximab resistant and sensitive cells. Novel predictive markers of cetuximab response are needed, and the present high throughput analysis of differences between cetuximab resistant and sensitive samples aims to provide such markers.

The gene copy number analysis found 39 genes that were amplified in cetuximab resistant cells and not in cetuximab sensitive cells. These genes were all present on two genomic regions, 11q22.1 and 5p13-15. Interestingly, no genes were found to be commonly deleted in resistant samples and normal in

sensitive cells. By qPCR verification, the YAP1 gene on 11q22.1 was found to influence cetuximab resistance. The mRNA expression of YAP1, which highly correlated to gene copy number, was also higher in cetuximab resistant samples than in sensitive. Three of the 20 cell lines harbored extensive YAP1 amplification, and these three were all cetuximab resistant. Finally, by siRNA modulation of YAP1 expression, we could restore cetuximab sensitivity in one of the two tested cell lines. These results show that YAP1 is a predictive marker for cetuximab resistance, although other markers play a role as well.

Gain of 11q22.1 has been previously reported in head and neck cancer (Snijders, Schmidt et al. 2005; Roman, Meza-Zepeda et al. 2008), and was found to predict poor survival in oral cancer subjects (Ambatipudi, Gerstung et al. 2011). YAP1 gene copy number gain was also recently reported as one of seven commonly altered genes in HNSCC (supplementary material, figure S1 Stransky, Egloff et al. 2011).

YAP1 is a transcription factor for genes involved in proliferation and anti-apoptosis. Its DNA-binding is hampered by phosphorylation, which is the main downstream effect of Hippo/Mst-1 signaling-induced contact inhibition. Contact inhibition is effected by E-cadherin mediated cell-cell contact, which was recently shown to be dependent on subcellular localization of YAP1 (Kim, Koh et al. 2011). The connection of YAP1 and the EGFR pathway has not been extensively studied; however, YAP1 activates the expression of the EGFR ligand amphiregulin (Dong, Gupta et al. 2011). HNSCC patients with high expression of amphiregulin are less likely to benefit from a combination of docetaxel and cetuximab (Tinhofer, Klinghammer et al. 2011). YAP1 also has shown a connection to radioresistance in medulloblastomas (Fernandez, Squatrito et al. 2011), raising the possibility that it might be an interesting drug target. Hypothetically, contact inhibition is hindered by excessive YAP1 levels, which leads to a continued transcription of proliferation genes. This is a possible

mechanism for deficient growth inhibitory effect of cetuximab in cells with YAP1 amplification.

In summary, amplification of 11q22.1 has been previously reported as a bad prognostic indicator in HNSCC. This study connects 11q22.1 amplification to cetuximab resistance, and proposes YAP1 as the driver gene of this unfavorable genetic defect.

GENERAL DISCUSSION

To be able to predict treatment response by the assessment of biomarkers is a common goal for many medical researchers. Many markers have been proven a connection to treatment response in HNSCC; however, none has made its way into clinical practice.

The establishment of microarrays and other high throughput analyses has inspired cancer researchers to new biomarker studies with an implemented systems biology approach, and the use of gene expression signatures has been proposed as possible prognostic or predictive markers. In the field of breast cancer, at least six multigene signatures are commercially available. These tests are qPCR or microarray based, ranging from 2 to 70 genes, and have demonstrated prognostic potential in deciding tumor grade (Espinosa, Vara et al. 2011). This also has impact on therapy decision, since patients with low grade breast cancer tumors are not assigned to chemotherapy. To date, no such gene expression signature is available for HNSCC prognosis or therapy deciding purposes. Many transcriptomic analyses in HNSCC have focused on expression differences in tumor versus normal tissue (Lallemant, Evrard et al. 2009). In order to establish a gene expression signature that predicts treatment response perhaps another strategy is needed. Preclinical models, such as cell line studies, could provide predictive gene signatures to be validated in clinical material (Sawyers 2008). To this end, the predictive gene expression signatures of cisplatin and radiation resistance presented herein awaits validation, which could be provided from testing in publically available data sets of clinical material.

Paper I and paper III of this thesis have different routes to a shared goal, i.e. identifying intrinsic radioresistance mechanisms. In paper I, mutation of p53 is shown to be important for radioresistance, together with EGFR expression and survivin expression. The main finding of paper III is that FN1 is predictive of a

radioresistant phenotype. The oncogenic potential of mutated p53 has been discussed elsewhere in this thesis, and is seemingly important in addition to loss of function of normal p53. Interestingly, mutant p53 is known to drive invasion by promoting integrin signaling (Muller, Caswell et al. 2009). This effect was prevented by EGFR- or integrin β 1-inhibition. Since FN1 signals through binding to integrins α 5 β 1, this is a possible mechanism that links the main findings of paper I and III.

The genotyping of tumor DNA has been useful for therapy response prediction, more specifically for resistance to EGFR targeted therapies. In addition to KRAS and BRAF mutations, PIK3CA mutation was recently associated with EGFR targeted therapy resistance, and PTEN expression was associated with response in KRAS wt metastatic colorectal cancer (Sood, McClain et al. 2012). In contrast to KRAS and BRAF mutations, which are rare in HNSCC, PIK3CA and PTEN mutations are among the most mutated genes in HNSCC (Stransky, Egloff et al. 2011). However, a study of 16 HNSCC cell lines concluded that PIK3CA or PTEN mutations did not influence cetuximab response (Kondo, Tsukuda et al. 2011). In addition to mutation status, gene copy number status can influence treatment response, and should therefore be carefully evaluated in biomarker testing (Sawyers 2008). EGFR copy number amplification is associated with response to EGFR small molecule therapies in lung cancer (Sharma, Bell et al. 2007), but this is not the case for HNSCC patients treated with cetuximab (Licitra, Mesia et al. 2011). With this background we decided to look for novel markers of cetuximab resistance with a gene copy number array comparing resistant and sensitive cell lines. We concluded that YAP1 is the probable driver gene of 11q22.1 amplification, which was previously shown to be a bad prognostic marker in HNSCC. Even though YAP1 amplification is not the exclusive resistance marker, our results suggest that all tumors that harbor extensive YAP1 amplifications are cetuximab resistant. These results await

verification on primary tumor material, and warrants additional studies on YAP1 deregulation in cancer.

Changing the design of clinical studies is a possible way of yielding reliable results of biomarkers in relation to treatment response. Biomarkers are rarely taken into account when appointing patients to treatment. Gonzales-Angulo et al. discussed how systems biology approaches in clinical trials will lead to smaller, shorter, and cheaper trials which will increase the success rate of cancer therapies (Gonzalez-Angulo, Hennessy et al. 2010). The clinical benefit of a drug is tested in trials, and the inclusion of biomarkers in phase II would enable the evaluation of biomarker quality along with the traditional drug efficacy testing (Beckman, Clark et al. 2011). A biomarker that could possibly be implemented in therapeutic decision making in HNSCC is HPV infection of oropharyngeal tumors. Since these tumors respond better to radiotherapy, chemotherapeutics could possibly be excluded without affecting success rates, and at the same time keeping the therapeutic side effects to a minimum.

The papers in this thesis take exclusively tumor cells into account. Apart from tumor cells, the tumor microenvironment also undergoes changes during malignant progression. The tumor stroma is built up from several cell types, the extracellular space, and vessels, all of which possess factors that can facilitate cancer progression (Hanahan and Weinberg 2011). The deregulated processes in the tumor cells are imperative to cancer progression and to treatment response. However, when extrapolating results, one should keep the potential influence of stromal factors in mind.

CONCLUSIONS

From the results presented in this thesis, the following conclusions can be drawn:

- The NNP model successfully combines markers on protein and genetic level to create a strong predictive model to intrinsic radiosensitivity
- A combination of EGFR expression, survivin expression and splice site or missense mutations in p53 is a panel of markers that strongly correlates to intrinsic radiosensitivity
- Expression of MMP-7 predicts intrinsic cisplatin sensitivity
- Expression of MMP-13 associates to intrinsic cisplatin sensitivity
- Expression of FN1 predicts intrinsic radiosensitivity
- Amplification of the YAP1 gene associates with intrinsic cetuximab sensitivity
- Modulation of YAP1 expression can alter cetuximab resistance

FUTURE PERSPECTIVES

This thesis addresses important questions that are not easily studied. Tumor resistance to treatment involves multiple interconnected pathways, and to extract a few regulating factors responsible for this resistance is challenging. Although these results are probably representative for HNSCC, future studies should aim at investigating the possible presence of these markers in primary tumor material and to evaluate them in relation to treatment response and survival.

Since the NNP model was established it has shown promising results in several *in vitro* studies, with larger material and a revised panel of markers involved in intrinsic radiosensitivity and intrinsic cisplatin sensitivity (Farnebo, Jedlinski et al. 2009; Farnebo, Jerhammar et al. 2011). Assessment of selected markers in a tumor bank is underway and these results are highly anticipated.

Papers II and III has a similar bioinformatic workflow, which resulted in transcriptional profiles representing intrinsic cisplatin sensitivity and intrinsic radiosensitivity, respectively. The value of these transcriptional profiles could be tested using a clinical material training set and clinically relevant parameters such as locoregional control or survival. Public databases should be consulted in order to find a dataset with these reported parameters. The single markers proposed in these papers should also be modulated in order to have extensive evidence for their functional relevance for cisplatin and radioresistance. The single markers that have functional importance could also be tested in the tumor bank, in order to establish the expression in primary tumor, and the possible relation to survival.

In the progress of screening cell lines for cetuximab sensitivity in paper IV, we found that two cell lines proliferated under cetuximab treatment. These cell lines should be analyzed for any common dysfunction underlying this phenotype. Key

findings should also be tested in patient material in order to evaluate if this potentially devastating deregulation exist in the clinic.

The finding that YAP1 amplification leads to cetuximab resistance is promising, since 11q22.1 amplification has already been coupled to worse prognosis in HNSCC. The presence of YAP1 amplification should ideally be tested in a tumor material of cetuximab treated patients, in order to evaluate this finding in its proper clinical setting.

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