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Predictive markers for treatment sensitivity in head and neck squamous cell carcinoma

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*To my beloved family
SIMON,
Lydia and Svante*

In God we trust, from others we demand data

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ABSTRACT

Head and neck cancer is the sixth most common cancer world wide. In Sweden approximately 850 new cases are diagnosed each year, and two thirds are men. The past decades of improved treatment strategies have unfortunately not significantly improved the five-year survival rates for this group of patients. Therefore, it is important to rapidly find combinations of new and strong predictive markers for treatment response. Different predictive markers have been investigated for decades, without succeeding in finding means to securely predict response to treatment. Models to combine markers are called for.

The aim of this thesis was to test multiple predictive markers on both gene and protein level to evaluate their predictive value for radiotherapy and cisplatin response. Furthermore, to combine, and correlate them to treatment response in order to extract the panel of markers that strongest correlated to the investigated treatment. Cell lines derived from 42 patients with head and neck squamous cell carcinoma (HNSCC) were used for protein quantification with Western blot and ELISA of the proteins survivin, Epidermal Growth Factor Receptor, Bcl-2, Bcl-X_L, Bax, Bad, Bak, PUMA, Heat shock protein 70, MDM2, p53, SMAD4, Cyclooxygenase-2, and Cyclin D1. The expression of the selected proteins was related to the mean expression of normal oral keratinocytes (NOK) from healthy individuals. Furthermore, mutations in the p53 gene, along with single nucleotide polymorphisms in the genes of p53, MDM2, FGFR4, XRCC1, XRCC3, XPD, and XPC were analysed. To allow a large number of predictive markers on both protein and gene level to be combined and correlated to treatment response, the number of negative points (NNP) model was introduced. Both correlations of sensitivity to radiotherapy and to cisplatin treatment was analysed among the cell lines. In the first paper, including nine cell lines, the panel of EGFR, survivin, and splice site/missense p53 mutations correlated strongest to radioresponse. In paper II, 42 cell lines were used and the combination of survivin, Bcl-2, Bcl-X_L, Bax, COX-2, and the p53 Arg72Pro polymorphism was found to most strongly correlate with radioresponse. In paper IV, the panel correlating strongest with cisplatin sensitivity consisted of EGFR, Hsp70, Bax, and Bcl-2 in combination with SNPs in the DNA-repair genes XRCC3 and XPD.

The predisposition of the FGFR4 Gly388Arg polymorphism for the development of HNSCC was investigated in paper III. DNA was isolated from 110 tumour biopsies, and restriction fragment length polymorphism analysis showed that 58% of the individuals in the control group carried the FGFR4 Arg³⁸⁸ allele, whereas the frequency in the tumour group was 45%. The Gly³⁸⁸ allele gave a significantly higher risk of developing HNSCC, suggesting Gly³⁸⁸ to be the risk allele for cancer development. Furthermore, a novel mutation was found in the FGFR4 gene. The influence of this new mutation is however unknown.

In conclusion, predictive markers for treatment sensitivity need to be combined to receive an accurate prediction of treatment response.

LIST OF PAPERS

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**, Fredrik Jerhammar, Linda Vainikka, Reidar Grénman, Lena Norberg-Spaak, and Karin Roberg (2008)
Number of Negative Points: A novel method for predicting radiosensitivity in head and neck tumor cell lines.
Oncology Reports 20:453-461
- II **Lovisa Farnebo**, Fredrik Jerhammar, Rebecca Ceder, Roland Grafström, Linda Vainikka, Lena Thunell, Reidar Grénman, Ann-Charlotte Johansson, and Karin Roberg (2010)
Combining factors on protein and gene level to predict radioresponse in head and neck cancer cell lines.
Submitted to Journal of Oral Pathology and Medicine
- III Anna Ansell, **Lovisa Farnebo**, Reidar Grénman, Karin Roberg, and Lena Thunell (2009).
Polymorphism of FGFR4 in cancer development and sensitivity to cisplatin and radiation in head and neck cancer.
Oral Oncology 45: 23-29
- IV **Lovisa Farnebo**, Adam Jedlinski, Anna Ansell, Linda Vainikka, Lena Thunell, Reidar Grénman, Ann-Charlotte Johansson, and Karin Roberg (2009)
Proteins and single nucleotide polymorphisms involved in apoptosis and DNA repair predict cisplatin sensitivity in head and neck cancer cell lines.
International Journal of Molecular Medicine 24:549-556

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ABBREVIATIONS

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
BER	Base excision repair
Bid	BH3 interacting domain death agonist
C/w	cells/well
Co-SMAD	Common mediator SMAD
COX-2	Cyclooxygenase-2
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DSB	Double strand break
EGFR	Epidermal growth factor receptor
FGFR4	Fibroblast growth factor receptor 4
Gy	Gray
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
Hsp70	Heat shock protein 70
IAP	Inhibitor of apoptosis protein
ICS	Intrinsic cisplatin sensitivity
IR	Intrinsic radiosensitivity
I-SMAD	Inhibitory SMAD
MeV	Mega-electron volt
MDM2	Murine double minute 2
MMP	Mitochondrial membrane permeabilization
MMR	Mismatch repair
NER	Nucleotide excision repair
NNP	Number of negative points
NOK	Normal oral keratinocytes
OR	Odds ratio
PUMA	p53 up-regulated modulator of apoptosis
RNA	Ribonucleic acid
R-SMAD	Receptor regulated SMAD
SGLT1	Sodium/glucose cotransporter 1
SF	Surviving fraction
SMAD	Mothers against decapentaplegic (MAD) and the <i>Caenorhabditis elegans</i> protein (SMA). The name is a combination of the two
SNP	Single nucleotide polymorphism
TGF- β	Transforming growth factor beta
TNM	Tumour - node - metastasis
Wrap53	WD40 encoding RNA antisense to p53
XRCC	X-ray repair cross-complementing

INTRODUCTION

Cancer

Cancer is a genetic disease that often takes decades to develop. Multiple independent steps are required to break down the complex regulatory pathways that maintain normal growth in a cell. When a single cell acquires a mutation in an oncogene or a tumour suppressor gene it gains a growth advantage over its neighbours, enabling cancer development. The number of cells from the originally mutated clone increases, and there is a great risk that a second mutation will occur that allows its offspring to grow even faster. This cycle continues when cells accumulate additional mutations that accelerate their growth and metastatic potential. Molecular analyses of oncogenes and tumour suppressor genes in tumours can predict not only the course of the disease, but also suggest appropriate treatment (Watson, 1999).

The incidence of cancer is increasing worldwide. It is believed that one third of all cancer cases could have been prevented if well known risk factors had been avoided. In the industrial countries, lifestyle factors are believed to be of greatest importance, whereas the cancer is primarily related to infections in the developing countries. Smoking is the most common lifestyle-related risk factor and despite the known hazard of smoking almost every third adult still smokes in Sweden today (Jaresand, 2008).

Cancer of the head and neck

Head and Neck cancer is the sixth most common cancer world wide and accounts for 6% of all cancer in adults (Parkin et al., 2005). The World Health Organisation predicts a continuing worldwide increase in incidence, extending into the next several decades (Bettendorf et al., 2004). 95% of all head and neck cancers consist of squamous cell carcinomas (HNSCC), which is the only patho-anatomic diagnosis studied in this thesis. The overall five year survival rate is around 50% for this group (Thomas et al., 2005) and the prognosis has not improved dramatically during the past 20 years (Forastiere et al., 2006), even though many attempts have been made to optimize treatment. In part, this is explained by the fact that at least 50% of the patients have an advanced disease at diagnosis (stage III or IV).

Diagnosis is based on thorough physical examination and endoscopy to assess the tumour macroscopically and to harvest tumour biopsies for microscopic evaluation. To localise the area of the primary tumour as well as the metastatic spread, various radiological techniques including computed tomography, magnetic resonance imaging, and positron emission tomography are used.

Lymphatic metastases are found mainly in the neck region. Distant metastases are rare at initial presentation (10%) and occur primarily in the lung (Ries, 2006). The risk to develop a second primary tumour in the oral cavity varies between 5-30%, and will most commonly appear within 3 years of the primary tumour (Jones *et al.*, 1995)

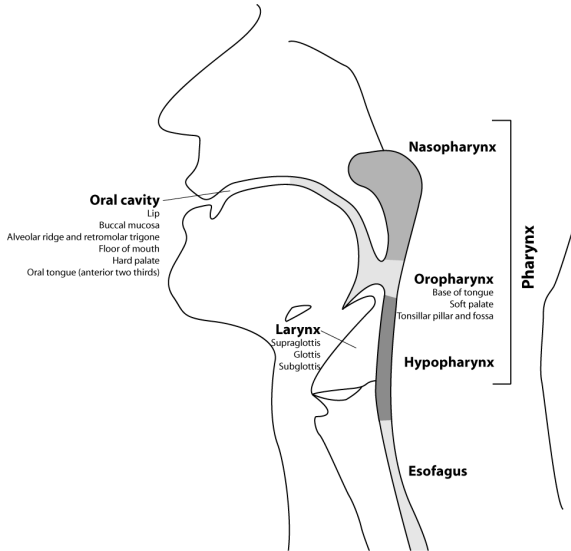


Figure 1
Regions where HNSCC occur.

In 2006, 1.7% of the Swedish population developed a cancer, and 843 new head and neck cancer cases were documented (Ferlay *et al.*, 2007). The male predominance of 3:1 is mainly explained by an overconsumption of tobacco and alcohol, a combination that gives a 50-fold increased risk to develop HNSCC (Blot *et al.*, 1988, Vineis *et al.*, 2004). Other risk factors for HNSCC are human papillomavirus infection (D'Souza *et al.*, 2007), low intake of fruit and vegetables (Pavia *et al.*, 2006), bad socioeconomic environment,

and poor dental health (Branchi *et al.*, 2003). The development of HNSCC was commented in a review from 2009 stating that HNSCC develops through an area exposed to carcinogens in combination with accumulation of genetic aberrations. Multicentric origin of cancer through field cancerisation is considered a vital factor in the recurrence or persistence of the disease after therapy (Makitie *et al.*, 2009).

Traditionally the typical patient with a head and neck tumour would be a 62-year old man who smokes, drinks, and has lived a hard life. However, over recent years a new group of patients has emerged where the patient instead can be a 32-year old woman, with no risk factors in terms of tobacco smoking and alcohol consumption. Interestingly, the 62-year old man is likely to have a better prognosis than the 30 year younger woman. It is speculated that a change in sexual habits after 1969 towards a more liberated sexuality, including oral sex, is one possible reason for the increase of head and neck tumours in the younger population. This increase in incidence could possibly be explained by the spread of human papillomavirus which increases the cancer incidence in the tonsillar region and the base of tongue (D'Souza *et al.*, 2007).

Treatment

Therapeutic decision making

Despite increasingly radical surgery, plastic reconstruction, and various combinations of radio- and chemotherapeutic treatments, the 5-year survival rates have remained disappointingly stable. Reliable tools for prediction of treatment outcome are sparse. Decision making is today largely based on the TNM-classification, which has been shown to be an insufficient predictor for treatment response. Furthermore, tumour grade (differentiation) rarely influence treatment decisions since no evidence of an association between grade and loco-regional control has been shown (Bettendorf et al., 2004).

Advanced T and N stages and large tumour volume are associated with a decrease in loco-regional control, an increase in distant metastasis, and a shorter disease-free survival. Early stages of cancer (stages I and II) are highly curable by surgery or radiotherapy alone (Shah, 2007), whereas advanced cancers (stages III and IV) are generally treated with surgery and pre- or post-operative radiotherapy, sometimes in combination with chemotherapy. Patients with identically staged tumours can, however, respond differently to therapy. This limitation of the TNM-classification to predict treatment outcome is likely to be due to its lack of biological consideration where the different characteristics of the tumour cells are not taken into account. Therefore, a system that enables prediction of a patients' response to therapy would allow for optimization of treatment outcome (Argiris et al., 2008, Silva et al., 2007). Identification of biomarkers that will guide treatment decisions and individualise the treatment of HNSCC patients would therefore be most welcome.

Surgery

The standard treatment for HNSCC is surgery. The possibility to cure patients with surgery, however, is limited by tumour size and the desire to maintain important functions such as swallowing and speech through organ preservation. Advances in reconstructive surgery, such as microvascular free-flaps, have substantially improved the functional outcome, although this has not affected the overall survival (Shah, 2007).

Radiation

Surgery is most often combined with pre- or post-operative radiotherapy in HNSCC. The radiotherapy aims at causing irreparable DNA damages. This results in cell cycle arrest, apoptosis, gene inactivation, reproductive failure, or terminal senescence of the tumour cells (Chen et al., 2007). Due to the fact that the overall survival rate has not

improved during the past 20 years with conventional radiotherapy, attempts have been made to enhance the effect of radiotherapy including intensity-modulated radiotherapy (Argiris et al., 2008), accelerated fractionation, and hyperfractionation (Horiot et al., 1992) although the results have not been revolutionary so far (Peters, 2007).

Many factors have been shown to affect the response to radiotherapy including haemoglobin level, smoking habits during radiotherapy, and tumour location (Silva et al., 2007). Side-effects following radiotherapy in the head and neck region include severe mucositis in the oral cavity, hoarseness, swallowing disorders, and local skin rashes.

Chemotherapy

Chemotherapy is reserved for patients with locally advanced HNSCC, to whom it is given in combination with radiation and sometimes surgery. The radiation and chemotherapy interaction was originally defined by Steel already in 1979 (Steel, 1979), but was confirmed showing an improved survival of 4% at 5 years in patients with non-metastatic HNSCC treated with chemotherapy concomitant to radiotherapy as compared to the group receiving radiation and/or surgery without chemotherapy (Pignon et al., 2000). Panels of chemotherapeutic agents are used in the clinic, including taxanes, anti-metabolites, and platinum containing compounds. Cisplatin, which belongs to the latter category, is regarded as the drug of choice in Linköping, if the patient's performance status allows for its addition.

Cisplatin has a cytotoxic effect with inhibition of the DNA-synthesis independent of cell cycle phase. As it enters the cell by diffusion (Rosenberg et al., 1969) the active metabolite reacts with cellular DNA to form inter- and intrastrand crosslinks causing inhibition of DNA replication and RNA transcription. Cisplatin induces DNA strand breaks and miscoding that are either repaired, mutagenic, or lethal, causing activation of apoptosis (Wilson et al., 2006). Side-effects are well known and include neutropenia, nephro-, neuro-, and ototoxicity as well as nausea and vomiting.

Chemoradiation

Combined treatment regimens often look promising in phase II trials but fail to show a treatment advantage in phase III trials (Eisbruch *et al.*, 2005, Haffty *et al.*, 2005, Henke *et al.*, 2003, Warde *et al.*, 2002). A large meta-analysis established that chemoradiation was slightly superior to radiotherapy alone. Chemotherapy given concomitantly to radiotherapy gave an absolute survival increase (8% higher at 5 years from diagnosis) although related to increased toxicity (Pignon et al., 2007). Another meta-analysis compared concomitant chemotherapy to induction chemotherapy and found an absolute benefit for concomitant chemotherapy of 6.5% at 5 years (Pignon et al., 2009).

When cisplatin is combined with radiotherapy, it results in severe mucositis in the oral cavity.

EGFR-targeted therapy

Targeted therapy is a promising field in cancer therapeutics, and the drug industry has launched an arsenal of compounds in recent years. These are often targeting growth factor receptors or their downstream signalling.

Cetuximab is a monoclonal antibody directed against the epidermal growth factor receptor (EGFR). It is the first molecularly targeted agent to receive positive survival data in HNSCC. Bonner *et al.* showed that radiotherapy in combination with cetuximab gave an increase in overall survival (49 months vs. 29 months) as compared to radiotherapy alone (Bonner *et al.*, 2006). When bound to the EGFR, cetuximab inhibits ligand binding, downstream signalling, and hinders EGFR-coupled gene expression (Jaramillo *et al.*, 2006, Li *et al.*, 2008). Depletion of antibody-bound EGFR from the cell surface is believed to be an important mechanism underlying cetuximab-induced growth inhibition in vivo (Sigismund *et al.*, 2005).

Cellular processes influencing treatment sensitivity

The body has many security check-points to avoid incorporation of pathological DNA in the reproduction of cells. Events with major influence on normal cellular regeneration such as apoptosis, cell cycle regulation, and DNA repair are central in keeping the genome intact. Cancer may occur when the balance between these events is disturbed. An altered function of these fundamental cellular processes is also likely to affect the treatment sensitivity.

Apoptosis

Cellular suicide, apoptosis, allows the organism to tightly control cell number and tissue size. A cell can self-degrade in order for the body to eliminate unwanted or dysfunctional cells. Apoptosis is initiated either from outside the cell (death receptor pathway/extrinsic pathway) or from the inside (mitochondrial pathway/intrinsic pathway). In both pathways, signalling results in activation of caspases, which execute apoptotic cell death. The morphologic characteristics of the apoptotic cell include chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and cell shrinkage. An immense advantage of apoptosis is that phagocytes engulf the apoptotic cells without causing an inflammatory response as opposed to necrosis (Zwaal *et al.*, 2005).

The extrinsic pathway (death receptor pathway) is engaged when a ligand binds to a cell-surface death receptor that transmits the apoptotic signal to the interior of the cell. This mechanism is used to eliminate unwanted cells in the body, including cancer cells or

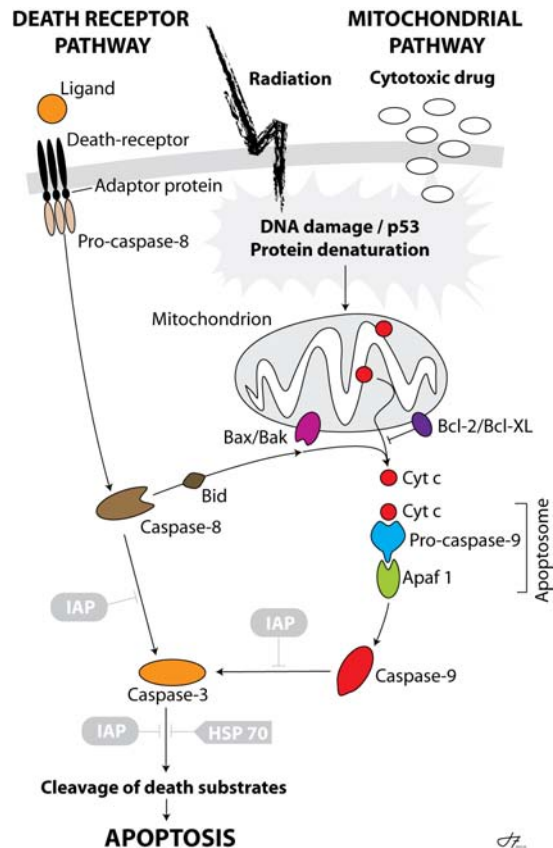


Figure 2
The death receptor (extrinsic) and mitochondrial (intrinsic) pathways of apoptosis.

infected cells (Falschlehner et al., 2007). Upon activation death receptors interact with an adaptor protein which in turn binds to caspase-8, forming the death-inducing signalling complex (Vangestel et al., 2009).

The intrinsic pathway (mitochondrial pathway) is activated by cellular stresses like starvation, ionizing radiation, DNA-damage, hypoxia, or exposure to various chemicals. This pathway is mainly controlled by the balance between pro- (e.g., Bax, Bak, Bid, and PUMA) and anti-apoptotic (e.g., Bcl-2 and Bcl-X_L) members of the Bcl-2 family. Upon an apoptotic stimuli Bax/Bak are activated leading to mitochondrial membrane permeabilisation and release of pro-apoptotic proteins to the cytosol (Garrido et al., 2006). One of these proteins, cytochrome c, triggers the assembly of the apoptosome causing activation of caspase-9 (Vangestel *et al.*, 2009). Caspases cause apoptotic cell dismantling by cleavage of multiple proteins leading to loss of cell structure and function.

The two pathways are inter-connected by Bid, a pro-apoptotic member of the Bcl-2 family. Caspases activated via the extrinsic pathway cleave Bid, generating a pro-apoptotic truncated form that engages the intrinsic pathway by promoting Bax/Bak-mediated mitochondrial membrane permeabilisation.

Many anti-cancer therapies eliminate cancer cells by the induction of apoptosis via either of these pathways. Therefore, alterations in expression or function of proteins controlling the process of apoptosis can highly influence patients' sensitivity to different anti-cancer treatments.

Cell cycle regulation

Human cells possess a proliferative capacity in vast excess of that required to meet the needs of normal cell growth and development (Andreff M, 2005). *In vivo*, normal human cells can divide as often as twice daily. A cell dividing at this speed would generate a cell number equal to the total amount of cells in a human body in two months. This dividing capacity is however, highly regulated in order to limit cell division to appropriate times (wound healing) and places (for example, organs with rapid cell turn over). The cell cycle includes the S phase, lasting around eight hours, in which DNA is replicated. Progression into G₂ takes place, where the cell rests and synthesises cellular constituents needed to support the next phase being the M phase. In this phase fully replicated

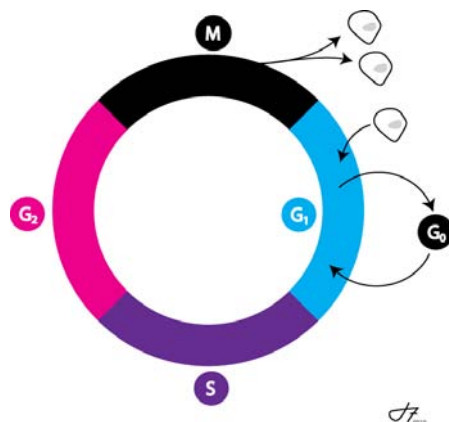


Figure 3
Cell cycle. G₁= Gap 1, G₀=Gap 0, S=Synthesis, G₂=Gap 2, M=mitosis

chromosomes are segregated to each of the two daughter nuclei, in mitosis. The M phase normally takes about one hour before the cell proceeds into the G_1 phase. The G_1 phase is the only phase that is highly variable in time, ranging from six hours to several days or longer (G_0). The cell cycle is tightly controlled; one product acts as a substrate for the next to ensure that no further step can take place unless the previous step was completed. In tumour cells; however, cell cycle checkpoints are relaxed so that the proliferation is speeded and the time for cell rest is decreased. Most anti-cancer therapies act by killing cells that divide rapidly, which is one of the main reasons why cancerous cells are more sensitive to such treatments than normal cells. Tumours are enriched in cells in S phase and the G_2 and G_1 phases are minimized. However, anti-cancer therapies also harm normal cells that divide rapidly such as cells in the bone marrow, digestive tract, and hair follicles, and result in the most common side effects including myelosuppression, mucositis, and alopecia.

DNA-repair

The number of spontaneous base damages per human cell per day is approximately 25,000 bases out of the 3×10^9 bases in the genome (Friedberg, 2001). The load of base damage from naturally occurring and environmentally related sources would be incompatible with life unless cells were endowed with specific mechanisms for repairing DNA damage. On nuclear DNA damage, normal cells activate cell-cycle checkpoints, upregulate genes involved in DNA repair, and initiate apoptotic cell death. A cell normally rests in G_1 if DNA damage is sensed. This arrest is induced to prevent the replication of damaged DNA. If cells are already in S phase, DNA replication is slowed down to allow time for repair. There are at least four pathways of DNA repair and the pathway used depends on the type of DNA damage.

1. Base excision repair (BER) operates on small lesions where a single damaged base is removed by base-specific DNA glycosylases (Lu et al., 2001). The abasic site is then restored by endonuclease action, DNA synthesis using the other strand as template, and ligation.
2. The nucleotide excision repair (NER) pathway repairs bulky lesions and involves at least four steps; damage recognition, unwinding of the DNA, removal of the damaged single-stranded fragment, and finally synthesis of DNA (Goode et al., 2002).
3. In the homologous recombination pathway double-strand breaks (DSBs) caused by exogenous agents like radiation are repaired (Khanna et al., 2001). DNA ends are resected and the exposed 3' single-stranded tails invade the double helix of

the homologous undamaged partner molecule. Strands are extended by DNA polymerase and then cross over to yield two intact DNA molecules.

4. In the mismatch repair (MMR) pathway replication errors caused by DNA polymerase errors are corrected (Kolodner et al., 1999).

In cancer cells, DNA repair mechanisms are dysfunctional due to the speeded cell cycle. Furthermore, cancer cells may not repair the damages as effectively as normal cells because of mutations in the repair genes, in some cases leading to increased treatment sensitivity.

Single Nucleotide Polymorphisms (SNPs)

In 2001, the sequence of the human genome was completed and it became clear that different individuals were >99% identical (Bond et al., 2005). The differences between people were about 4.5 million SNPs distributed throughout the genome, in coding and non-coding regions. These differences contribute to inter-individual traits that define every human as unique. To be defined as a SNP the variant allele must exist at a single base pair position within the genomic DNA in normal individuals, and the least frequent allele must have an abundance of a minimum of 1% in the population (Brookes, 1999), thus a polymorphism is not automatically a SNP. SNPs are thought to play an important role in many common diseases including diabetes, mental illness, cardiovascular disease, and cancer. It is the combination of several SNPs in key genes along with environmental factors, rather than a SNP alone that determine whether an individual will be predisposed to develop a certain disease or not. SNPs in certain genes are 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.

Predictive markers

A useful predictive marker foretells the response to therapy, as measured by remission rate, and guides treatment decision, but does not assess future biological behaviour of the tumour (Akervall, 2005).

It is highly unlikely that a single factor would be a robust enough predictor of therapy response in all tumours (West et al., 2005). Therefore, numerous potential biomarkers need to be analysed in order to identify strong predictive markers for treatment sensitivity which could be combined to securely predict treatment outcome. In this thesis, a number of factors, that according to the literature could influence the response to radio- or chemotherapy, were evaluated for their usefulness as biomarkers predictive for therapy response in head and neck cancer.

Survivin

Survivin, which is a member of the inhibitor of apoptosis protein (IAP) family, regulates two essential mechanisms in the cell; it blocks apoptosis by inhibition of caspase activation and it is a regulator of mitosis.

It is not fully understood mechanistically how the IAPs inhibit apoptosis, however it has been suggested that IAPs bind to and inhibit activated caspases-3, and -7, which are the main effector caspases in the signalling of apoptosis (Tamm et al., 1998). However this model was challenged by the observation that survivin lacked the structures (present in other IAPs) that mediates binding of caspases. Later findings indicated that survivin together with co-factors inhibited caspase-9, but not -3, and -7 (Marusawa *et al.*, 2003). Thus, survivin prevents apoptosis, although its mechanism of action may be more sophisticated than direct caspase inhibition and could involve cooperation with other molecules (Mita *et al.*, 2008).

It is also still unclear how survivin regulates cell mitosis. By confocal microscopy, survivin was found to be absent in the more part of interphase, but present towards the end of G2, and high in M phase (Caldas et al., 2005) where among other functions survivin in association with regulators of cytokinesis is essential for proper chromosome segregation (Lens *et al.*, 2006).

Survivin is expressed at high levels during fetal development, but is rarely seen in normal adult tissue. It is often overexpressed in human cancers including HNSCC (Lippert et al., 2007), and 90% of the cell lines used in this thesis showed overexpression of survivin as compared to NOKs. Survivin is generally accepted as a significant independent prognostic indicator of poor outcome (Fukuda et al., 2006). However, there is contradictory data indicating that a high survivin expression in oral squamous cell carcinomas predicts an increased 5 and 10 year overall survival (Freier et al., 2007). It was

speculated that these contradictory results are dependent on the subcellular localisation of survivin. In normal cells, survivin's regulating function of mitosis is predominant. The up-regulation of survivin expression in cancer cells seems to be independent of the cell cycle. However, an increase of survivin's antiapoptotic role is suggested. Therefore, the subcellular localisation of survivin in tumours (cytoplasmic and nuclear) may indicate survivin activity and serve as a predictive marker (Engels *et al.*, 2007).

Survivin promotes tumour-associated angiogenesis by inhibition of endothelial cell apoptosis (Lo Muzio *et al.*, 2005). Down-regulation of survivin has been shown to sensitize tumour cells to apoptosis (Chawla-Sarkar *et al.*, 2004) and halt tumour progression by blocking angiogenesis (Altieri, 2003).

Malignant cells with an overexpression of survivin fail to execute apoptosis which makes them resistant to both radio- and chemotherapy (Dean *et al.*, 2007). Furthermore, survivin is a target for anti-cancer drug discovery (Altieri, 2008, Capalbo *et al.*, 2007). Anti-survivin therapies are likely to have adverse effects on normal cells (Fukuda *et al.*, 2001), however, studies on mouse xenografts showed a significant reduction of human breast and prostate cancer cell growth without apparent toxicity (Plescia *et al.*, 2005).

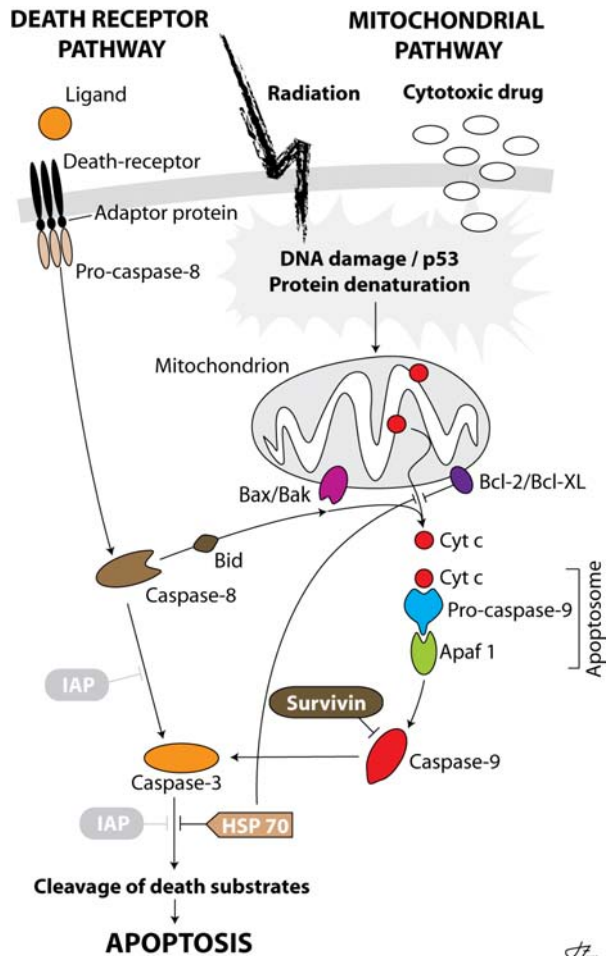


Figure 4

The role of survivin (IAP), the Bcl-2 family, and Hsp70 in downstream signalling of apoptosis.

Members of the Bcl-2 family

B-cell lymphoma 2 (Bcl-2) is the founding member of the Bcl-2 family of apoptosis regulator proteins (Tsujimoto *et al.*, 1984). Bcl-2 family proteins either induce (pro-

apoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c and other apoptogenic factors from the intermembrane space of the mitochondria into the cytosol. In the cytosol, cytochrome c triggers activation of the executioners of apoptosis, the caspases (Fesik *et al.*, 2001). Proteins of the Bcl-2 family can be divided into three functional groups: 1) proteins that permeabilise the mitochondrial outer membrane such as Bax and Bak 2) proteins that trigger Bax/Bak-mediated mitochondrial membrane permeabilisation (MMP), like Bad and PUMA, and 3) proteins that prevent MMP such as Bcl-2 and Bcl-X_L.

It is the balance between the anti- and the pro-apoptotic Bcl-2 family members that determines whether apoptosis takes place or not (Strasser *et al.*, 2000). The expression of both pro- and anti-apoptotic proteins of the Bcl-2 family has been reported to be regulated by the tumour suppressor protein p53 (Miyashita *et al.*, 1994, Nakano *et al.*, 2001).

Bcl-2 has been implicated in a number of cancers, including melanoma, breast, prostate, and lung carcinomas, as well as other diseases like schizophrenia and diabetes. This supports a role for decreased apoptosis in the pathogenesis of cancer. Bcl-2 overexpression was noticed in 13% of HNSCC tumours (Wilson *et al.*, 2001), and is considered to be more extensively overexpressed in advanced and aggressive cancer. It is also thought to be involved in resistance to conventional cancer treatment. It has sometimes been associated with a more favourable outcome irrespective of treatment schedule, however it is more often described as associated with an increased radiation resistance, especially when combined with a low expression of Bax (Haffty *et al.*, 2003). Radioresistance was observed in tumour cells with an overexpression of the anti-apoptotic protein Bcl-2, or an underexpression of the pro-apoptotic Bax, Bad, Bak, and PUMA (Condon *et al.*, 2002, Guo *et al.*, 2000).

The anti-apoptotic proteins Bcl-2 and Bcl-X_L and the pro-apoptotic members Bax, Bak, PUMA, and Bad were studied in this thesis.

Heat shock protein 70 (Hsp70)

When cells are exposed to elevated temperatures or other types of stress, heat-shock proteins (Hsp) are induced and help cells to cope with these stresses (De Maio, 1999). Hsps are named according to their molecular weight. For example, Hsp70 refer to heat shock protein with 70 kilodaltons in size (Li *et al.*, 2004). Hsps are highly expressed in cancerous cells and are essential to their survival by protecting them from changes in their environment (Lee *et al.*, 2007). An upregulation of Hsp70 is found in HNSCC, as compared to normal epithelium (Weber *et al.*, 2007).

Among the heat shock proteins both Hsp70 and Hsp27 have been implicated in tumourigenesis and chemoresistance, probably via the prevention of apoptosis (Lee *et al.*, 2007). Hsp70 expression has been associated with radioresistance, since inactiva-

tion of Hsp70 increased residual DNA DSBs after exposure to radiation and lead to increased apoptosis. This supports a role of Hsp70 in radiation-induced DNA damage repair (Pandita et al., 2009). Overexpression of Hsp70 is believed to protect cells from apoptosis after radiation and help malignant cells survive the treatment. High expression of Hsp70 has also been associated with resistance to chemotherapy (Garrido et al., 2006).

The epidermal growth factor receptor (EGFR)

The EGFR is a member of the ErbB2-family and a cell surface receptor found primarily on cells with epithelial origin. On ligand binding, the inactive EGFR monomer associates with a second EGFR molecule, or alternatively other members of the ErbB2 family, forming a homodimer or heterodimer, respectively. Dimerisation stimulates intracellular protein kinase activity resulting in autophosphorylation of tyrosine residues in the catalytic domain of EGFR (Downward et al., 1984). This autophosphorylation elicits downstream signalling leading to DNA synthesis, cell proliferation (Oda et al., 2005), enhanced migration, and adhesion (Wells, 1999).

EGFR overexpression or overactivity has been associated with a number of epithelial cancers and is associated with invasion and metastasis (Argiris et al., 2008), poor prognosis, and treatment resistance (Nicholson et al., 2001). EGFR overexpression was found in 30% of all epithelial tumours (Kuan et al., 2001) and in 30% (Bettendorf et al., 2004) to 90% of HNSCC (Argiris et al., 2008). It is considered an indicator of poor prognosis (Ang et al., 2002, Grandis et al., 1993, Hitt et al., 2005, Shin et al., 2001) and resistance to chemotherapeutic drugs (Ang et al., 2002).

Blocking of EGFR by tyrosine kinase inhibitors results in response rates around 10-20% in HNSCC (Cohen et al., 2003). Expression levels of EGFR in cancer has been correlated to prognosis; however, not with responsiveness to tyrosine kinase inhibitor treatment (Arteaga, 2002). These findings suggest that EGFR may contribute to the progression of cancer also by mechanisms independent of its kinase activity. For ex-

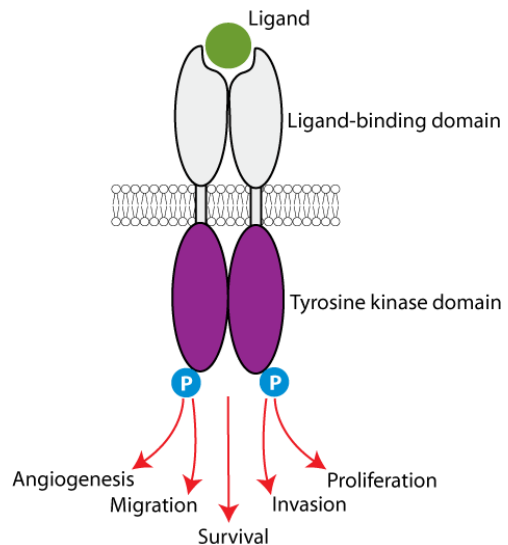


Figure 5
The EGF-receptor and its intracellular mechanisms.

ample, EGFR facilitates glucose transport into cells by association with and stabilisation of sodium/glucose cotransporter 1 (SGLT1) (Weihua *et al.*, 2008). This interaction between EGFR and SGLT1 is believed to coordinate cell growth and division with nutrient uptake. Disruption of the complex may affect intracellular glucose levels and this may in turn influence a tumour cells ability to withstand chemo- or radiotherapy (Engelman *et al.*, 2008).

Interestingly, EGFR has been shown to translocate into the nucleus upon receptor activation. In the nucleus, EGFR is involved in many cellular processes, such as DNA synthesis, and DNA repair (Wang *et al.*, 2006), and transcription of genes associated with cell proliferation, tumour growth, and metastasis (Lo *et al.*, 2005). Both ionising radiation and cisplatin are known to induce translocation of EGFR into the nucleus. Nuclear EGFR was associated with an increased activity of DNA-dependent protein kinase, an enzyme taking part in DNA DSB-repair. A mutation in the nuclear localisation signalling region of EGFR released EGFR-induced cisplatin resistance. Re-introduction of the nuclear localisation signal allowed EGFR to re-enter the nucleus and the cells regained resistance to cisplatin, due to restored DNA-repair activity (Hsu *et al.*, 2009).

Cyclin D1

Cyclins are regulators of cyclin-dependent kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the coordination of each mitotic event. Cyclin D1 forms a complex with cyclin-dependent kinases 4 and 6. This complex functions as a regulatory subunit required for cell cycle G1/S transition.

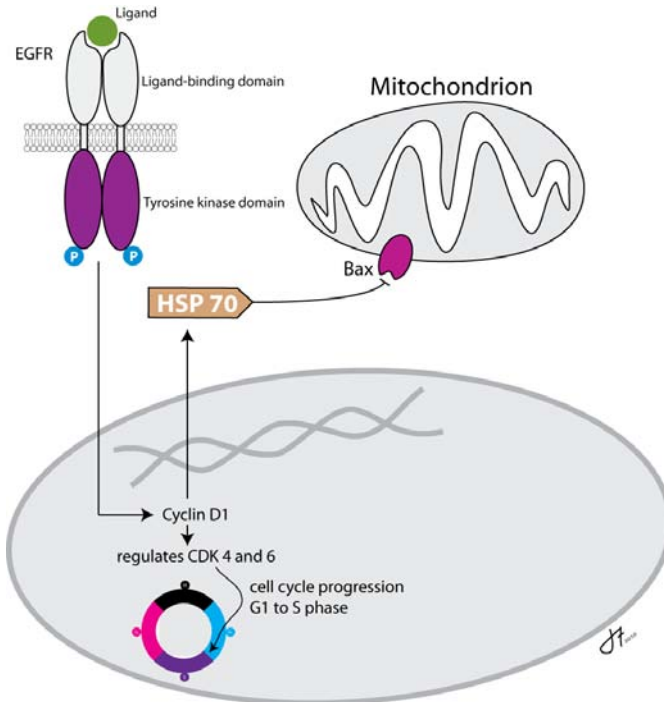


Figure 6

Cyclin D1 is activated downstream of EGFR and stimulates cell cycle progression and apoptosis.

Furthermore, cyclin D1 promotes cellular senescence, apoptosis, and tumorigenesis (Roue *et al.*, 2008). The relation between elevated levels of cyclin D1 and prognosis of HNSCC, and prediction of treatment response is not entirely clear. High levels of cyclin D1 have been correlated with poor radio-response (Milas *et al.*, 2002). But contradictory results exist and show that cyclin D1 overexpression was associated with radio-sensitivity in squamous cell carcinomas (Shintani *et al.*, 2001). Cy-

clin D1 overexpression was found in 36-40% of HNSCC (Koontongkaew *et al.*, 2000, Nakahara *et al.*, 2000), and the expression of cyclin D1 is regulated by EGFR (Mandic *et al.*, 2009, Milas *et al.*, 2002). In cyclin D1-producing cells Hsp70 was accumulated intra-cellularly and inhibited the pro-apoptotic protein Bax, in order to delay and impede apoptosis (Roue *et al.*, 2008). Thus, overexpression of cyclin D1 within tumour cells could increase resistance to cancer treatments by prevention of apoptotic cell death.

Cyclooxygenase-2 (COX-2)

COX-2 is a key enzyme in the conversion of arachidonic acid to prostaglandins and interleukins. COX-2 expression is induced by various factors and is linked to carcinogenesis, tumour growth, and metastatic spread by its product prostaglandin H_2 , which is converted into prostaglandin E_2 that in turn can stimulate cancer progression. Tumours expressing high levels of COX-2 showed a decreased radiation sensitivity (Shin *et al.*, 2005, Terakado *et al.*, 2004) and resistance to chemotherapy (Koki *et al.*, 1999) due to reduced susceptibility to apoptosis (Thomas *et al.*, 2005). Inhibition of COX-2 has been shown to increase radiation sensitivity (Kishi *et al.*, 2000, Milas, 2001, Pyo *et al.*, 2001) and to sensitize tumour cells to chemotherapeutic agents (Saha *et al.*, 2003). Consequently, inhibition of COX-2 may have benefit in the treatment of COX-2 overexpressing cancers (Menter *et al.*, 2010).

SMAD4

SMADs are a group of proteins that modulate the signalling following transforming growth factor beta (TGF- β) receptor activation (Wrana, 2000). TGF- β is a protein that controls proliferation, cellular differentiation, and other functions in most cells. It acts as an antiproliferative factor in normal epithelial cells and at early stages of oncogenesis (Heldin *et al.*, 1997). However, in later stages of tumour progression TGF- β promotes tumour growth and metastasis. Due to genetic alterations such as SMAD mutations tumour cells fail to respond adequately to the TGF- β signal. Moreover, tumour cells often overexpress TGF- β which, in a paracrine manner, leads to changes in the tumour microenvironment that support tumour progression. SMAD4, often in complex with other SMADs, acts as a transcription factor that regulates the expression of certain genes (Massague *et al.*, 2005). There are three classes of SMADs:

1. The receptor-regulated SMADs (R-SMAD) including SMAD1, SMAD2, SMAD3, SMAD5, and SMAD8/9 (Wu *et al.*, 2001).
2. The common-mediator SMAD (Co-SMAD) which includes only SMAD4, which interacts with R-SMADs to participate in signalling (Shi *et al.*, 1997).
3. The antagonistic or inhibitory SMADs (I-SMAD) which include SMAD6 and SMAD7, which block the activation of R-SMADs and Co-SMADs (Itoh *et al.*, 2001).

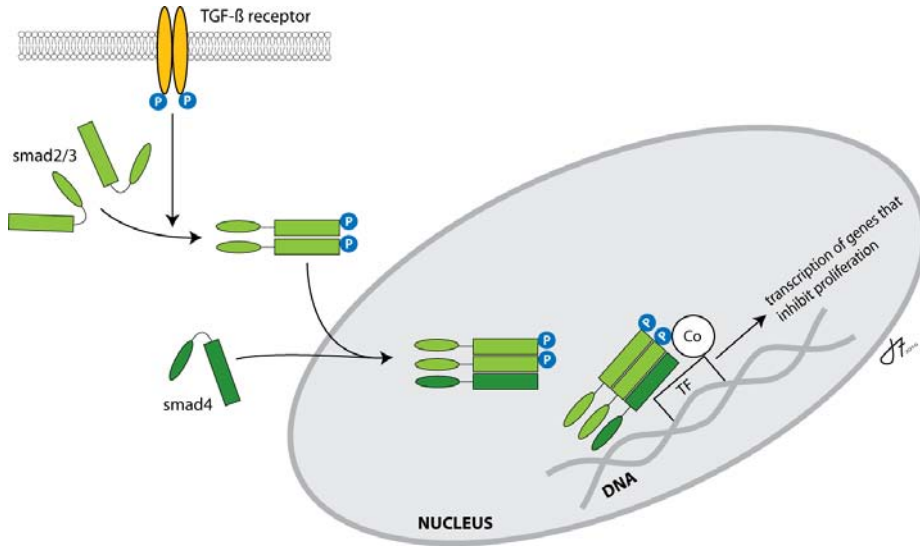


Figure 7

SMAD complexes are formed on TGF- β receptor activation and enter the nucleus to activate transcription factors involved in cell proliferation, apoptosis, and differentiation. TF=transcription factor, Co=co-activator/repressor.

Upon TGF- β receptor ligation R-SMADs are activated and interact with SMAD4 (Grady, 2005). This complex translocates to the nucleus and takes part in the regulation of gene transcription. The SMAD signalling is deregulated in various cancer types including HNSCC (Xie *et al.*, 2003). It is possible that simple inactivation of the TGF- β activated SMAD signalling pathway is sufficient to change gene expression that favours tumour formation (Grady, 2005). It is not fully understood how SMAD4 affects treatment response. SMAD4 expression is correlated to an increased response to various cytostatic drugs in esophageal cancer as well as in colorectal cancer (Boulay *et al.*, 2002, Puhlinger-Oppermann *et al.*, 2010). However, high levels of SMAD4 have also been associated with an increased resistance to chemotherapeutic drugs (Ji *et al.*, 2007). The relation to radiotherapy response is also still unclear.

p53

Due to the central role of p53 in cell cycle control and apoptosis the p53 protein, as well as mutations and SNPs in the p53 gene, were analysed in this thesis.

The purpose of the p53 signal transduction pathway is to ensure the fidelity of the duplication process of DNA in the cell (Bond *et al.*, 2004, Bond *et al.*, 2005), and stress signals dramatically increase the half-life of the p53 protein (Appella *et al.*, 2001). There are three major outcomes of the p53 stress response: 1) cell cycle arrest in G1 to S, 2) cellular senescence, and 3) apoptosis. The p53 protein concentration increases in

a cell and it becomes an active transcription factor. This is at least in part mediated by inactivation of a key negative regulator of p53, MDM2. P53 acts as a break to the cell cycle and enables DNA repair before cell division. If the DNA is not repaired effectively, apoptosis is instead initiated.

Mutations in the core domains (exons 5-9) of the p53 gene can result in DNA faults being incorporated in the genome, and progress to cell division instead of apoptosis. Both mice and humans harbouring an inactivating mutation in one allele of the p53 gene develop tumours very early in life and at dramatically high frequencies. Accordingly, somatic inactivating mutations of the p53 gene are found in over 50% of all human tumours (Balz *et al.*, 2003) and occur within at least 60% of HNSCC (Ahomadegbe *et al.*, 1995).

Naturally occurring polymorphic genetic variants in critical locations of the p53 pathway might underlie the variation seen between individuals in their susceptibility to cancer and the progression of their disease (Alberts, 2004). A SNP in codon 72 changes Arg to Pro, where the Arg/Arg genotype induces apoptosis, and suppresses malignant transformation more efficiently than the Pro/Pro genotype (Thomas *et al.*, 1999). The association between the p53 Arg72Pro SNP and the risk for oral cancer has been debated and no correlation between the SNP and risk for HNSCC was found (Summersgill *et al.*, 2000, Tandle *et al.*, 2001). In 2007, Kuroda *et al* suggested that the Pro/Pro genotype increases the risk for oral cancer in non-smokers and worsens the prognosis in this group (Kuroda *et al.*, 2007).

The p53 protein is capable of either arresting the cell cycle or inducing apoptosis (Zhan *et al.*, 1994). Deregulated expression of proteins controlling apoptosis may suppress the apoptotic signal that would normally follow upon DNA damage. Thus make cells with high levels of p53 protein more resistant to treatments that depend on apoptosis to kill off tumour cells, including radiation and cytotoxic drugs. Cells containing mutated p53 or SNPs in the p53 gene, lack functional p53 tumour suppressor activity, and may lead to high expression of the p53 protein or dysfunctional protein, which also can result in a loss of the normal apoptotic signal that would follow upon anti-cancer treatment, and can thus increase treatment resistance.

Murine double minute 2 (MDM2)

The oncogene MDM2 is a negative regulator of the p53 tumour suppressor. The MDM2 protein binds to p53 and promotes its transport out of the nucleus to the cytosol thereby hindering the transcriptional activity of p53.

Furthermore, it functions as an ubiquitin ligase that targets the p53 protein for degradation in the proteasome. P53 regulates the MDM2 expression, forming a negative feedback loop (Michael *et al.*, 2003). In most physiologic conditions, MDM2 maintains p53 at low levels to enable normal cell growth and development, but overexpression of

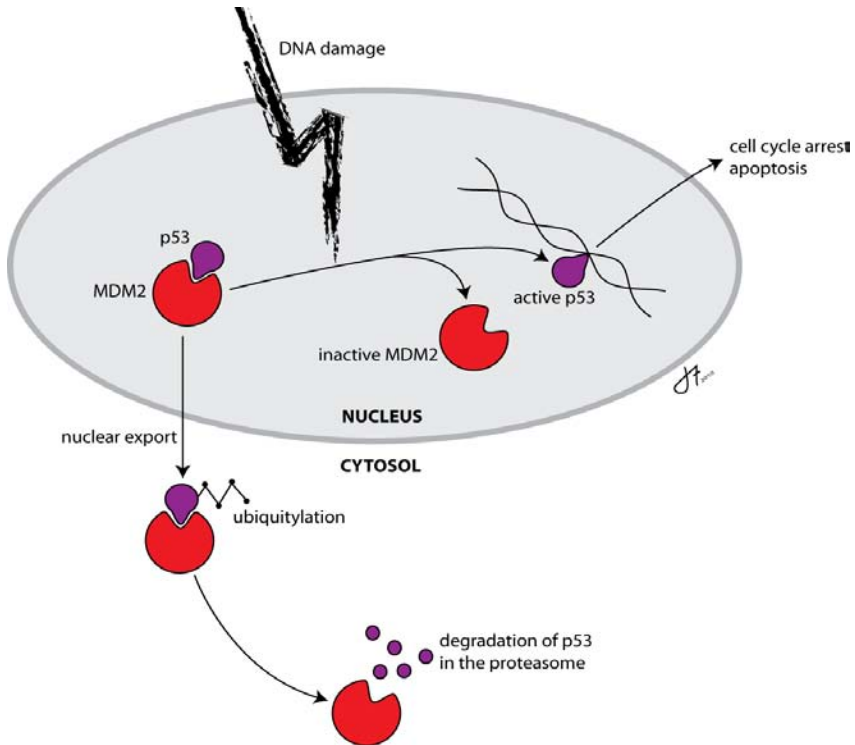


Figure 8

MDM2- the negative regulator of p53. MDM2-p53 complexes are exported from the nucleus and p53, ubiquitinated and degraded in the proteasome. On DNA damage MDM2 is inactivated and p53 can induce transcription of genes involved in cell cycle arrest and apoptosis.

MDM2 may inhibit p53 function and make damaged cells escape the cell cycle checkpoint and thereby become carcinogenic (Bond *et al.*, 2005, Haupt *et al.*, 1996). Mice with reduced levels of MDM2 are small, lymphopenic, and radiosensitive with increased levels of apoptosis, depending on their elevated p53 function (Mendrysa *et al.*, 2003). Conversely, mice with a 4-fold increase in MDM2 have tumour development in 100% of the cases (Jones *et al.*, 1998).

Cells with SNPs in the MDM2 gene can, depending on the SNP, lack the balance between p53 and MDM2. The usual labelling of SNPs is that the number represents the codon where the SNP is localized. However, the MDM2 T309G SNP is located in the promoter (and not the coding) region and therefore the number instead represents the nucleotide 309 in intron 1, T-to-G. Cells with the G/G genotype, generally have a higher expression of MDM2 protein, and thereby a lower apoptotic response. As a consequence, a higher number of cells continue to live and propagate, inducing tumour formation (Bond *et al.*, 2004). In individuals with the Li Fraumini syndrome with low wild type p53 activity and with the MDM2 309 SNP (G/G or G/T), the patients developed

cancer 10-12 years earlier than the group of patients with Li Fraumini syndrome and T/T genotype in this SNP (Bond *et al.*, 2005).

Since cells with the SNP 309 G/G, generally have higher levels of MDM2 protein, in turn suppressing p53 function and thereby apoptosis, it is speculated that tumour cells with this SNP are more resistant to treatments that are dependent on apoptosis for cell death.

Fibroblast growth factor receptor 4 (FGFR4)

FGFRs consist of four closely related genes (FGFR1-4) and belong to the receptor tyrosine kinase family. FGFRs consist of three extracellular immunoglobulin-like domains, a single membrane-spanning segment, and a cytoplasmic tyrosine kinase domain (Powers *et al.*, 2000). There are 23 closely related members in the FGFR ligand family, the fibroblast growth factors. All four FGFRs can interact with a various number of ligands suggesting that there is a high evolutionary conservation within the FGFR family (Chen *et al.*, 2005). The extracellular portion of the protein interacts with fibroblast growth factors, setting off a cascade of downstream signalling, influencing mitosis and differentiation. The FGFR family members differ from one another in their distribution throughout the body.

Since FGFRs are involved in normal cellular processes including cell growth, tissue development, differentiation, angiogenesis, tissue repair and survival, any deregulation of its function can lead to developmental defects, and cancer. FGFRs have been implicated in many human cancers e.g. cervix, bladder, and breast (Streit *et al.*, 2004). Faults in FGFR1, 2, and 3 have been linked to Crouzon syndrome, Jackson-Weiss, and achondroplasia (Meyers *et al.*, 1995). The specific function of FGFR4 is unknown, but an increased expression has been found in many human cancers. A SNP changing the sense codon 388 from glycine to arginine was identified (da Costa Andrade *et al.*, 2007). The FGFR4 Gly388Arg polymorphism is present in about 50% of the population, and is causally connected to aggressive tumour progression and metastasis, but has no clear role in the risk for tumour formation (Bange *et al.*, 2002). The Arg³⁸⁸ allele is associated with poor prognosis in breast, and colon cancer (Bange *et al.*, 2002), sarcomas (Morimoto *et al.*, 2003), and HNSCC (da Costa Andrade *et al.*, 2007, Streit *et al.*, 2004). Furthermore, the Gly³⁸⁸ allele seems to have a protective effect in cancer progression, proposing that the amino acid exchange gives a loss of tumour suppressor function.

The FGFR4 Arg³⁸⁸ allele has been associated with resistance to adjuvant therapy in primary breast cancer (Thussbas *et al.*, 2006). Patients with the Gly³⁸⁸ allele were favoured when treated with adjuvant therapy supporting the loss of tumour suppressor function theory in FGFR4 carrying the Arg³⁸⁸ allele. When a high FGFR4 expression was noted in combination with the Arg³⁸⁸ allele a significantly reduced survival was ob-

served as compared to the Gly³⁸⁸ allele (Streit *et al.*, 2004). Furthermore, the Arg³⁸⁸ allele predisposed for distant metastasis and late recurrences (da Costa Andrade *et al.*, 2007). The involvement of FGFR4 in the response to radiotherapy still remains unclear. However, it would be highly interesting to shed light on this mechanism since it is likely that the FGFR4 Gly388Arg polymorphism is not only involved in the risk of developing HNSCC, the recurrence rates, and the response to adjuvant therapy, but could also be involved in the response to radiotherapy.

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

Since the head and neck region is continuously exposed to exogenous as well as endogenous factors with potential danger to the DNA, it is relevant to believe that DNA repair genes may influence treatment sensitivity in HNSCC. Changes in DNA repair genes may affect an individual's susceptibility to HNSCC and also the response to therapy and prognosis of the disease (Carles *et al.*, 2006).

Xeroderma pigmentosum complementation group C (XPC) is a key protein in recognizing damaged DNA and initiates the NER pathway. More than a hundred polymorphic variants in the XPC gene have been identified. One of the most common ones is the Ala499Val polymorphism that has been widely studied and correlated to an increased risk of developing lung, bladder, breast, esophageal, skin, and head and neck cancer (Francisco *et al.*, 2008). However, the Ala⁴⁹⁹ allele was also found to be protective against colorectal- and endometrial cancers (Weiss *et al.*, 2005). The genetic variants may result in an altered DNA repair capacity, and thereby influence both the risk of cancer development and treatment response. To date the genotype-phenotype correlation has not been established for most polymorphisms, including those of the XPC gene. However, both hetero- and homozygous variant genotypes of the XPC Ala499Val SNP conferred significantly lower radiation-induced DNA damages than the wild type (Zhu *et al.*, 2008).

Also XPD takes part in the NER pathway of DNA repair. Mutations in the XPD gene can give rise to repair- and transcription defects (Evans *et al.*, 1997). The risk for HNSCC development in relation to XPD has been debated. Some found a tendency that the XPD Lys751Gln polymorphism gave an increased risk of HNSCC (Sturgis *et al.*, 2002, Sturgis *et al.*, 2000). However, in a Korean material no association was found between XPD polymorphisms and the risk of developing HNSCC (Ji *et al.*, 2010). The XPD Lys751Gln polymorphism had no association with response rate to cisplatin in non-small cell lung cancer (Kalikaki *et al.*, 2009, Yao *et al.*, 2009).

The X-ray repair cross-complementing group 1 (XRCC1) protein plays an important role in the BER pathway of DNA repair. After excision of a damaged base, it stimulates endonuclease action and acts as a scaffold in the restoration of the site (Vidal *et al.*, 2001). SNPs in the XRCC1 gene have been associated with a significantly increased risk

for lung, prostate, and esophageal cancer. However, their role in the development of HNSCC has been debated. Some studies point towards no association to risk of developing HNSCC for a single SNP in the XRCC1 Gln399Arg (Li *et al.*, 2007), however an increased risk for HNSCC if the SNPs Arg194Trp and Gln399Arg both were present (Kowalski *et al.*, 2009) others point towards an increased (Ramachandran *et al.*, 2006, Sturgis *et al.*, 1999) or a decreased risk for HNSCC in the presence of the XRCC1 Gln399Arg polymorphism (Huang *et al.*, 2005). This polymorphism has been related to a better response to cisplatin treatment in non-small-cell lung cancer (Giachino *et al.*, 2007).

XRCC3 functions in the homologous DNA DSB repair pathway and positive associations between SNPs in this gene and the development of cancer have been observed (Winsey *et al.*, 2000). For HNSCC, results are once again conflicting. Some argue that there is no increased risk for HNSCC with the XRCC3 Thr241Met polymorphism (Huang *et al.*, 2005). Matullo *et al* found a possible protective effect for the development of lung cancer (Matullo *et al.*, 2006) which is in agreement with other results showing that the risk to develop HNSCC with at least one variant allele in the XRCC3 Thr241Met polymorphism was significantly decreased as compared to wild type XRCC3 (Magnussen *et al.*, submitted). For esofagogastric cancer it was shown that the Met241Met genotype was associated with a better survival after cisplatin treatment than Thr241Thr and Thr241Met (Font *et al.*, 2008), however in non-small-cell lung cancer no relation was found between SNPs in the XRCC3 gene and response to cisplatin (Zhou *et al.*, 2010). A significant association was observed between the surviving fraction at 2 Gy and the XRCC3 Thr241Met polymorphism indicating that individuals with the variant allele could be more susceptible to radiation (Alsbeih *et al.*, 2007).

Other predictive markers

In this thesis we evaluated a number of selected factors for their usefulness as predictive markers. The factors were selected primarily because they had previously been shown to be associated to treatment sensitivity. There are plenty of other factors that could be valuable predictive markers, some of which have been recognised during later years. To mention a few of particular interest;

- Human papillomavirus (HPV) is a very important predictive marker for therapy outcome in tonsillar- and base-of-tongue cancers and indicates favourable response to radiotherapy (Sedaghat *et al.*, 2009). The protein p16 which is considered a surrogate marker for HPV infection is a cyclin-dependent kinase inactivator that slows down progression of the cell cycle. Genetic variations like loss of heterozygosity have been reported in high frequency in HNSCC (Coon *et al.*, 2004), and was associated with de-

creased survival (Ambrosch *et al.*, 2001) and distant metastasis (Namazie *et al.*, 2002), and could therefore also be of interest as a predictive marker for treatment outcome.

- WD40 encoding RNA antisense to p53 (*WRAP53*) is a natural antisense gene to p53. Transcription of *WRAP53* gives rise to p53 antisense transcripts that interacts with the 5'untranslated region of p53 mRNA and thereby protects them from degradation. *WRAP53* transcript regulates both basal p53 levels and p53 action upon DNA damage (Farnebo, 2009, Mahmoudi *et al.*, 2009). The *WRAP53* gene also gives rise to a protein. The *WRAP53* protein was recently identified as a new subunit of the telomerase enzyme and essential for telomerase elongation in human cancer cells (Venteicher *et al.*, 2009). Telomerase function is related to the intrinsic radiosensitivity of human oral cancer cells (McCaul *et al.*, 2008). The close connection between *WRAP53*, p53 and telomerase makes *WRAP53* interesting to evaluate as a predictive marker in HNSCC.

- Fibronectin 1 has been shown to be expressed at higher levels in radioresistant cells of head and neck cancer origin, as compared to radiosensitive cells, and could therefore be a possible biomarker for radioresistance (Jerhammar *et al.*, in press, Cancer Biology & Therapy). Blood plasma levels of fibronectin was shown to be elevated in 66% of head and neck cancer patients (Warawdekar *et al.*, 2006). However, no correlation was found between plasma levels and stage of the disease, indicating that fibronectin holds a potential role as a predictive marker for radiotherapy response rather than as a tumour marker.

AIMS OF THE THESIS

The general objectives of this thesis were to find strong predictive markers for treatment response, to find a model to combine factors on both protein and gene level and furthermore, to test this model for radio- and cisplatin- therapy in cell culture.

More specifically, the aims of the studies were:

1. To establish a method by which multiple predictive factors on both protein and gene level could be combined to predict treatment sensitivity in HNSCC cell lines
2. To find a combination of predictive markers that correlates to intrinsic radiosensitivity using the NNP model in cell lines
3. To investigate the predisposition of the FGFR4 Arg388Gly polymorphism for the development of HNSCC, and furthermore, to examine if the FGFR4 Arg³⁸⁸ allele is associated with resistance to cisplatin or radiotherapy.
4. To find a combination of predictive markers that correlates to intrinsic cisplatin sensitivity using the NNP model in cell lines.

MATERIAL AND METHODS

Ethical aspects

The Nuremberg Code, which is the fundamental guideline for ethical committees in Sweden, states that all studies should have voluntary consent from the participating subjects, any risks for the participants should be minimized, and subjects should be free to discontinue trials at any time. The researchers are obliged to interrupt a trial if suspicion arises that participation could be dangerous. Furthermore, the research should be of benefit to the society in general (Markman et al., 2007). The studies in this thesis were approved by the Human and Ethical Committees at the Faculty of Health Sciences, Linköping, Sweden, and all patients included in this thesis have given their informed consent to the biopsies taken for scientific use.

Cells and culture conditions

These studies have been performed on 42 cell lines derived from HNSCC, kindly provided by Professor Reidar Grenman at Turku University, Finland. All cell lines were cultured in Dulbecco's Modified Eagle's Medium, supplemented with glutamine, non-essential amino-acids, penicillin-G, streptomycin, and fetal bovine serum, and tested free from mycoplasma contamination by DAPI staining. Cells were incubated in humidified air at 37°C, and subcultured weekly. Due to different problems with cell culturing including bacterial infection, and irregular growth patterns among some of the cell lines, 42 cell lines were used in paper II, 36 in paper III, and 39 in paper IV. In paper I nine of the 42 cell lines were selected according to their sensitivity to radiation, to represent the different parts of intrinsic radiosensitivity (IR).

The Linköping HNSCC biobank

From January 2004 and on, tumour biopsies have been collected from HNSCC patients at Linköping University Hospital (approved by the ethical committee of Linköping University Hospital, Dnr 03-537). The biopsies are harvested at the ear-nose and throat department during diagnostic procedures. Tumours with other patho-anatomical diagnoses than squamous cell carcinoma are saved in the biobank, although excluded from the research in this thesis. One half of the material is snap-frozen and stored at -70°C, while the other is cut into small pieces and placed in cell culture flasks for experiments. Any remaining material is fixed in formaldehyde and paraffin-embedded and thereby preserved from degradation. The bank as of 2010-09-01 contained 250 frozen biopsies, 28 cell lines, and 40 paraffin-embedded tumour pieces. This is a unique set of consecutive tumours, since we have access to the patients' medical charts and can

provide information about risk factors, clinical response to therapy, and outcome. Future research will primarily be performed on material from our own biobank, since we can then follow the patients clinically as well as in the lab.

Assessment of intrinsic radiosensitivity

Cells were harvested with trypsin, counted, and diluted to a standard stock solution of 4167 cells/ml. With this concentration, 2 cells/well (c/w) in control plates was reached when 200 μ l of cell suspension was applied per well. The number of cells plated per well was adjusted to the plating efficiency of each cell line, and according to the expected cell kill as follows: control, 2 (c/w); 0.75 Gy (Gy), 3 c/w; 1.25 Gy, 4 c/w; 2.50 Gy, 8 c/w; 5.00 Gy, 10 c/w; 7.50 Gy, 16 c/w. Single cell suspensions were plated immediately into 96-well culture plates. The plates were incubated at 37° C for 24 h to allow cells to attach before irradiation.

The plates were then irradiated with 4MeV photons generated by a linear accelerator, delivering a dose-rate of 2 Gy/min. After 4-weeks incubation the number of positive wells, containing living coherent colonies of at least 32 cells were counted. Surviving fraction (SF) as a function of radiation dose was fitted by a linear quadratic equation, and the area under curve (AUC) was obtained by numerical integration (Fertil et al., 1984).

$$SF = \frac{(\text{no. positive wells} / \text{no. plated cells}) \times \text{no. plated cells in control}}{\text{no. positive wells in control}}$$

The AUC value equals the radiation dose at which 50% of the cells died. Thus, a low AUC value indicates that the cells die at lower radiation doses, and hence are radiosensitive. Cell lines with higher AUC values require higher radiation doses to die and are considered more resistant to radiotherapy (Erjala *et al.*, 2004, Grenman *et al.*, 1989, Pekkola-Heino *et al.*, 1995). The AUC values of the 42 cell lines which equals the IR-value, varied from 1.4-2.6.

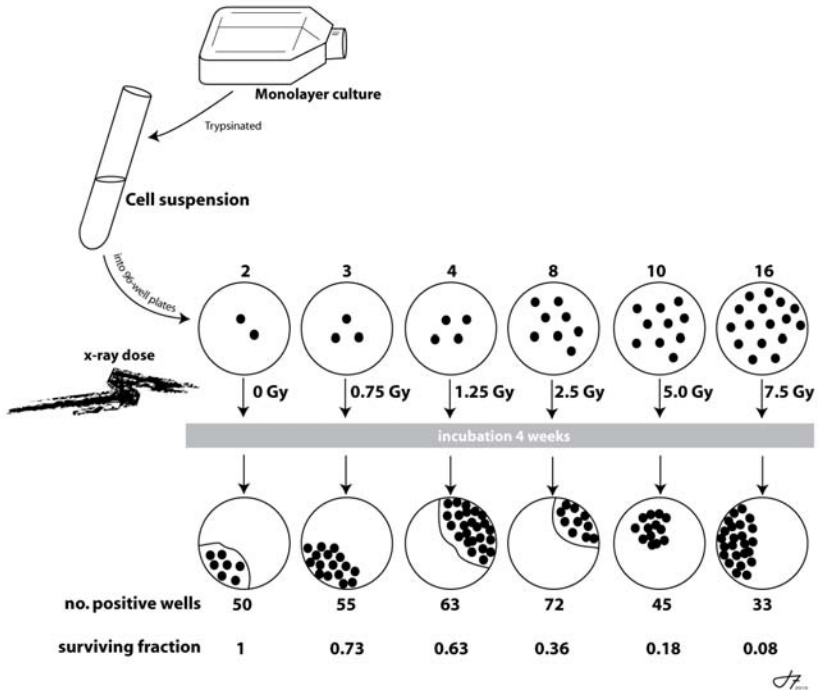


Figure 9

A schematic illustration of the 96-well plate clonogenic assay in testing the intrinsic radiosensitivity. A standard stock solution was used and further diluted to achieve the planned cell number per well. The number above each well represents the number of cells plated per well. Modified from K. Erjala, Thesis no. 717, Turku University, Finland

Assessment of intrinsic cisplatin sensitivity

To determine the intrinsic cisplatin sensitivity (ICS), tumour cells were seeded into six-well plates at cell densities ranging from 200-400 cells/cm² depending on their plating efficiency. After 24 h, cultures were exposed to cisplatin (1 µg/ml) for 1 h. The cells were then incubated for another nine days before formalin fixation, Giemsa staining, and counting of colonies containing 32 cells or more. The ICS values for different cell lines varied between 0-1, where an ICS of 1 equals 100% survival, as compared to untreated controls. All cell lines were exposed to cisplatin twice in triplicate using two different batches of fetal calf serum, and the mean value was used for statistical analyses. The highest variation in ICS value between the experiments with different serum batches was +/- 0.1.

Western blot

Western blot is a semi-quantitative method by which specific proteins can be detected. The name western blot is a play on the name Southern blot, a technique for detection of DNA developed by Edwin Southern. Western blot was used in paper I to analyse the expression of 14 different proteins. Cells were sampled from culture flasks and lysed

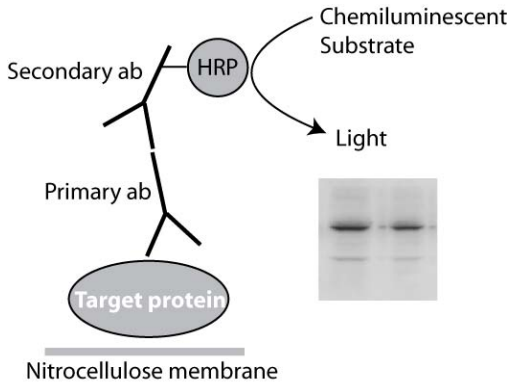


Figure 10
Schematic picture of the Western blot analysis.

*J*₂₀₁₀

before the protein content was determined using the method described by Lowry *et al* (Lowry *et al.*, 1951) to ensure that an equal amount of protein was added to each well on the polyacrylamide gel. An electrical field was then applied causing the proteins to separate according to molecular weight. The proteins were then transferred on to a nitrocellulose membrane, and detected using primary antibodies. Horse-radish peroxidase-conjugated secondary antibodies were added, catalysing the conversion of

chemiluminescent substrate to light in proportion to the amount of protein in the sample. Autoradiographic film was used to visualise the proteins.

ELISA

ELISA is a quantitative method for determination of the expression of specific proteins. This method was used in papers II and IV to analyse the expression of 7 different proteins. The total protein concentration was determined using the method described by Lowry *et al* (Lowry *et al.*, 1951) and the amount of specific proteins was analyzed by means of different commercially available ELISA kits. In brief, a capturing antibody was added to the plates, and non-specific binding sites were blocked using blocking buffer containing bovine serum albumin before the antigen-containing samples were added. Then a biotinylated detection antibody, which binds specifically to the antigen of interest, was added followed by streptavidin-conjugated to

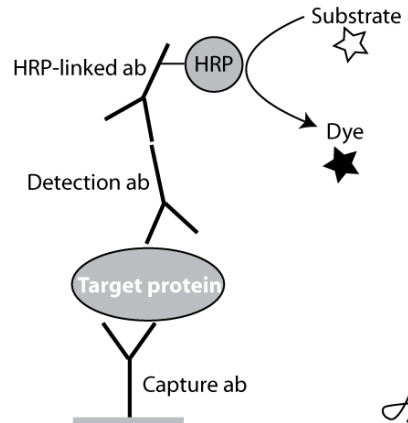


Figure 11
Schematic picture of the ELISA analysis.

*J*₂₀₁₀

horseradish peroxidase and the substrate solution containing the color reagent tetramethylbenzidine. Thereafter, stop solution was added and the optical density at 450 nm was analysed using a microplate reader. The amount of the detected protein was determined using a standard curve and correlated to the total amount of protein. All analyses were performed in triplicate and the mean values were used for further calculations.

Restriction fragment length polymorphism (RFLP)

RFLP is a genetic analysis which is used for DNA profiling. Here, RFLP was used to identify specific SNPs in the genes of p53, FGFR4, XPC, XPD, XRCC1, and XRCC3. The method is based on the use of restriction enzymes that cut the PCR-amplified DNA in specific regions, resulting in unique DNA fragments. These fragments are separated according to their length by gel electrophoresis, where smaller fragments migrate further than larger ones. They are then visualized by ethidium bromide and the various staining patterns obtained are characteristic to specific genotypes.

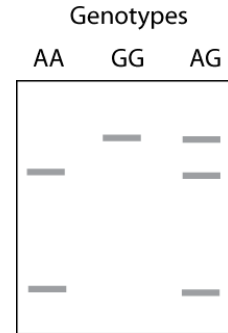


Figure 12
The result in a RFLP

Pyrosequencing of MDM2

A fragment of the MDM2 gene was amplified using a biotinylated reverse primer and, for the real-time sequencing of the PCR products and SNP analysis a pyrosequencing system was used. Single-stranded DNA was isolated from the PCR reaction using streptavidin-coated beads and the Pyrosequencing Vacuum Prep Workstation and was then transferred into a 96-well plate. The sequencing primer was annealed to the single-stranded DNA by heating and allowing it to cool to room temperature. The plate was then transferred to the pyrosequencing system where the nucleotides were added in a predetermined dispensation order. The system consisted of four enzymes and two substrates, where briefly, DNA polymerase incorporated a nucleotide and the pyrophosphate that was released initiated a reaction where a luciferase-driven light, proportional to the number of nucleotides bound, was detected and shown as a peak in the pyrogram.

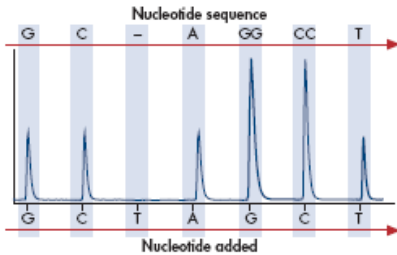


Figure 13
The result in a pyrosequencing.

The system consisted of four enzymes and two substrates, where briefly, DNA polymerase incorporated a nucleotide and the pyrophosphate that was released initiated a reaction where a luciferase-driven light, proportional to the number of nucleotides bound, was detected and shown as a peak in the pyrogram.

Single stranded conformation analysis (SSCA)

SSCA is an efficient method for screening of mutations in multiple samples, and provides a sensitive way to identify a small population of mutated cells within a large population of normal cells. SSCA was performed in Linköping for detection of mutations in exons 5-8 of the p53 gene and a similar method was used in Turku, Finland, for screening of exons 2-11. In SSCA, samples were marked in a PCR reaction by radioactively labeled nucleotides and separated on a polyacrylamide gel.

The pattern of separated bands was visualized using an x-ray film. Samples containing only one set of DNA strands resulted in two bands on the film, while samples containing DNA from both normal cells and tumour cells carrying a mutation generated four bands. The bands representing mutated DNA were excised, eluted, and used as template in a secondary PCR and sequenced in order to determine the exact location of the mutation.

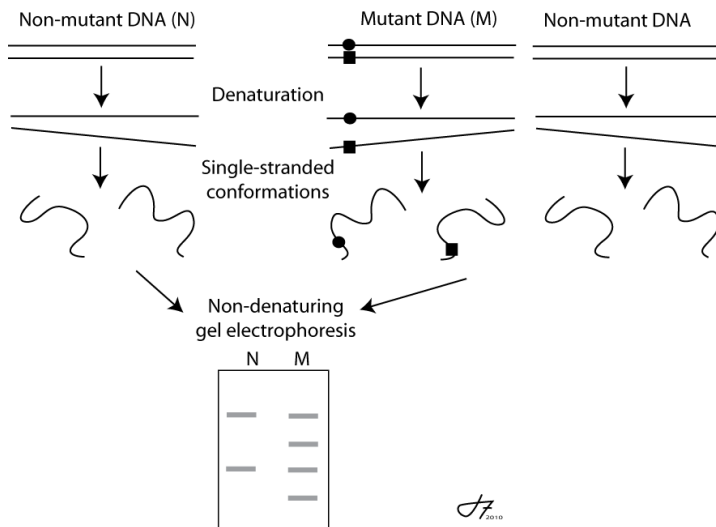


Figure 14
The principle behind the SSCA method

Number of Negative Points (NNP)

The NNP model was introduced to enable the combining of factors on both protein and gene level. The system was called the NNP since it describes the combination of different factors acting negatively on the host of the tumour i.e. the patient. The expression of proteins, mutations, and SNPs was analysed in HNSCC cell lines. If the expression of a protein was changed more than 1.5-fold as compared to the mean value for normal oral keratinocytes (NOK), the protein generated one point in the NNP system. Below that level it received zero points. If the expression was changed 4.5-fold or more the cell line got 2 points, and 3 points was given to cell lines with a 7.5-fold change or more. This scale worked in both directions, thus, overexpression of for example the pro-apoptotic protein Bax, which is associated with improved treatment sensitivity, and considered beneficial for the patient generated negative points in the NNP system and reduced the total NNP sum. For each p53 mutation, as well as for each SNP cell lines were given one point. In the NNP system the cell lines received a total score, the NNP sum, representing the potential sensitivity/resistance to treatment of each tumour cell line. We then combined factors in all possible ways to receive the blend of factors that strongest correlated to treatment response.

Statistical methods

Papers I, II, and IV: Pearson's correlation test was used to calculate the correlation to IR, or ICS, for single factors, as well as combinations of factors. The factor which on its own had the strongest correlation to IR or ICS was used as a starting point for coming correlation analyses. When a combination of two factors increased the correlation to IR or ICS, as compared to only one of them, both factors were kept for further calculations. If such a combination decreased the correlation that factor was not included in further calculations. When all factors were tested in combinations, a panel was extracted. In order to verify these results, all data were also analysed using a multivariate analysis, independently of the results received in the Pearson's analyses. Both methods concluded the same panel of markers.

Paper III: SNPs were tested for deviation from Hardy-Weinberg equilibrium using χ^2 -tests, as was the association between the frequency of the polymorphism and development of HNSCC. Overall survival was shown in Kaplan-Meier tables and the significance of the differences in survival rates between patients with different genotypes was assessed with log-rank test.

Papers II, III, and IV: Mann-Whitney U-test was used to analyse the impact of different SNPs for radio- and cisplatin sensitivity.

RESULTS AND DISCUSSION

Results paper I

The aim of paper I was to find biomarkers predictive of radioresponse and, furthermore, to establish a method which could combine multiple factors on both protein and gene level to increase the possibility to predict the radiosensitivity of HNSCC cell lines. We evaluated if a panel of predictive markers arranged in the NNP system alone or combined, correlated to IR. The combination of factors with the strongest correlation to IR was then extracted.

Nine HNSCC cell lines were selected to represent the whole range of IR varying from 1.4 to 2.6, with an average IR of 1.9. The IR value for each cell line was determined using a 96-well plate clonogenic assay. The expression of EGFR, survivin, COX-2, Bcl-2, Bcl-X_L, Bax, Bak, Bad, PUMA, Hsp70, Smad4, Cyclin D1, MDM2, and p53 was investigated by Western blot analysis. The expression of each protein was then related to the mean expression of the same protein in NOKs from healthy individuals.

Polymerase chain reaction- single strand conformation analyses were performed to identify p53 mutations, in the nine cell lines. The p53 mutations were then according to the type of mutation, arranged into three groups, type 1 (all mutations), type 2 (splice site and missense mutations), and type 3 (loss of transcript mutations). Among the nine cell lines in this paper, no mutations other than splice site/missense mutations or loss of transcript were present.

Correlation analyses were performed to conclude whether a single protein or mutation was associated with radioresponse. None of the factors alone correlated to the IR; however, the EGFR expression showed a clear tendency ($r=0.620$, $p=0.075$) to influence the treatment sensitivity. EGFR was then kept as a starting point when combinations of factors were analysed. A specific factor was kept for further analysis if the correlation to IR improved by adding that factor to EGFR. If however, a factor decreased EGFRs correlation to IR then it was not kept among the panel of factors with the strongest correlation to treatment response. When EGFR expression was combined with the expression of survivin, the combination of those two proteins correlated significantly to the IR ($r=0.880$, $p=0.002$). Each protein was then analysed together with the combination of EGFR and survivin. EGFR and survivin in combination with Bak, Hsp70, and Smad4 showed the strongest correlation to IR ($r= 0.886$, $p=0.001$).

To enable simultaneous evaluation of mutations and proteins, the NNP model was introduced. This model arranges both nominal scales (mutations) and ratio scales (protein values) into an interval scale (NNP). This was done in order to make western blot bands with highly varying intensity, as well as all-or-none results from mutation analy-

ses somewhat comparable, and possible to combine. When p53 mutations and proteins were arranged in the NNP model, a combination of all the investigated proteins and all mutations, showed a significant correlation to IR ($r=0.826$; $p=0.006$). This correlation was however, not as strong as the previous correlation found when combining only proteins from the western blot analyses. Furthermore, this paper aimed at identifying the combination of factors with the strongest correlation to IR and therefore Pearson's correlation test was once again used to extract such a panel. A multivariate analysis was then performed independent of the Pearson's correlation test, and verified the combination of factors initially found; EGFR, survivin, and splice site/missense p53 mutations, had the strongest correlation to IR ($r=0.990$, $p<0.0001$).

Discussion paper I

In the first study of this thesis, nine cell lines representing different parts of the IR spectrum were investigated. Considering the heterogeneity of tumours it is unrealistic to believe that a single predictive marker could securely predict treatment outcome in all patients. Single markers can be either weak or strong predictors of treatment response. However, even strong predictors need to be combined with other strong factors in order to reliably predict treatment response. The overall biomarker profile of a tumour will be indicative of its response to a specific treatment, rather than the expression of single markers (Buffa *et al.*, 2004). Attempts to combine factors are called for in the literature (Hanash *et al.*, 2008, Thomas *et al.*, 2005), however, such studies are very difficult to construct.

The NNP system was invented to enable correlation analysis on data derived from different types of assays combined. In this paper we have worked with protein expression in tumours assessed by western blot and genetic alterations through p53 mutation analysis. Because the relative densitometric values (ARD-values) from the western blots varied over a great range between different proteins, and since we wanted to evaluate both protein and gene changes in the same model, we needed to transform our data in such a way that all types of factors could fit into the same model. Since western blot is a semi-quantitative technique it is not the exact ARD-value that is of main interest, but rather the relative over- or underexpression as compared to NOK. Furthermore, as the ARD-values were rearranged into an interval scale in the NNP system we received the possibility to combine predictive factors at genetic level along with the protein expressions, and in addition a more relevant description of the level of protein expression was probably given.

In the NNP system all factors were presumed to be of equal importance although they may have different biological influence on treatment response. It is however necessary to make this assumption in order to create a simple model like the NNP model,

where the aim is to combine factors. However, it remains uncertain, which factor is of more importance for treatment sensitivity.

The combination of EGFR, survivin, Bak, Smad4, and Hsp70 correlated only slightly stronger to the IR than did EGFR and survivin alone. This implies that Bak, Smad4, and Hsp70 were not strong predictive markers of radioresponse. Furthermore, when they were arranged in the NNP system Bak, Smad4, and HSP70 did not increase the correlation to IR, indicating that they are of less importance than EGFR and survivin.

P53 mutations are very common in human cancer, the most common mutation being missense mutations (80%) (Levine, 1993). Mutations in the p53 gene often lead to a loss of the normal function of the p53 protein, but also a gain of function where cells acquire genomic instability, if the mutation is a DNA contact mutation (Yamazaki *et al.*, 2003). However, Hoffman *et al* found no association between the type of p53 mutation and the sensitivity to radiotherapy (Hoffmann *et al.*, 2008). In order to analyse whether different p53 mutations had varying effects on the IR the p53 mutations were classified into three groups. A loss of transcript can imply that no protein is produced at all and in this limited material three cell lines carried loss of transcript mutations and those did not show any impact on the treatment sensitivity. Splice site/missense mutations however, present in six of the cell lines improved the correlation to IR when combined with EGFR, and survivin. A speculation is that a splice site or missense mutation in the gene coding for the p53 protein alters the function of the protein extensively so that normal function is lost, which is advantageous for the continuation of tumour growth. The results in this paper furthermore indicated that the presence of splice site/missense mutations had a negative impact on radiosensitivity.

Results paper II

The aim of paper II was to find a panel of biomarkers that, when combined, show a strong correlation to radioresponse. The NNP model, introduced in paper I as a novel method to predict treatment outcome, was used for evaluation of combinations of predictive markers on both protein and gene level.

In this paper, 42 cell lines, including the cell lines investigated in paper I, were analysed with respect to their expression of seven proteins, four SNPs, and p53 mutations. The IR values were analysed with a 96-well clonogenic assay. Factors from paper I with the greatest influence on IR, (EGFR and survivin) were included, along with factors that in the literature were of particular interest in radiation response. The expression of EGFR, survivin, Bax, Bcl-2, Bcl-X_L, COX-2, and Hsp70 was quantified using ELISA, and related to the expression in NOKs. The p53 Arg72Pro, MDM2 SNP309, XRCC1 Arg399Gln, and XRCC3 Thr241Met polymorphisms are all believed to have an impact on the radioresponse and were thus investigated, along with p53 mutations.

Among the 42 cell lines investigated survivin was overexpressed in 38 (90%). A low overexpression was seen in 50% of the cell lines, an intermediate in 14%, and as much as 26% had a large overexpression. Survivin was found to significantly correlate to IR (Pearson's correlation test; $r=0.357$, $p=0.02$).

EGFR was overexpressed in 57% of the cell lines where a high overexpression was noted in four cell lines. COX-2 was upregulated in 21%, and 19% exhibited a low overexpression. Only one cell line (UT-SCC-2) had an intermediate change in expression, and none of the cell lines had a large change as compared to NOK. Hsp70 was overexpressed in all but six cell lines (86%). None had large changes in Hsp70 expression, while three had an intermediate change. The anti-apoptotic proteins Bcl-2 and Bcl-X_L were overexpressed only in 5% and 12% of the cell lines, respectively, and the overexpression in all of these cases was low. The pro-apoptotic protein Bax was underexpressed in 50% the cell lines, and overexpressed in two (5%), but Bax never had more than a low change as compared to NOK. None of the above proteins was significantly correlated to IR.

The wild type and variant alleles of the p53 Arg72Pro, MDM2 SNP309, XRCC1 Arg399Gln, and XRCC3 Thr241Met polymorphisms were investigated regarding their correlation to radiosensitivity, however, no association between these SNPs and radioresponse was observed. 90% of the cell lines were found to carry one or more mutations in exons 5-8 of the p53 gene. 10/42 (24%) was deletions or loss of transcript, and the remaining 28/42 (67%) were splice site/missense mutations. No correlation to IR was observed either the mutations were analysed separately or in combination.

To test if combinations of markers increased the correlation to IR, protein expression, mutations and SNPs were scored according to the NNP system, where each pro-

tein generated 0-3 points depending on the level of expression as compared to NOK, while each mutation and SNP received one point. The NNP for survivin alone significantly correlated to IR when analysed with Pearson's correlation test ($r=0.368$, $p=0.017$), and the strongest correlation was obtained when combining survivin, Bax, Bcl-2, Bcl-X_L, COX-2, and the p53 Arg72Pro polymorphism ($r=0.553$, $p<0.001$). A multivariate analysis was carried out independently of the Pearson's test and confirmed the combination of predictive markers with the strongest correlation to IR.

Discussion paper II

This study was performed on a substantially larger material, 42 cell lines, as compared to paper I ($n=9$). We extended our panel of possible predictive markers to include certain SNPs with potential influence on the radioresponse. In paper I we had chosen cell lines representing different segments of the intrinsic radiosensitivity scale; three cell lines had a very high IR value, i.e. were radioresistant, three had a low IR value, and the remaining three an intermediate IR value. In paper II, most of the 42 cell lines had an intermediate IR value and the fraction of cell lines with high or low values decreased in proportion. In fact, apart from the three highly radioresistant cell lines in paper I no additional cell line had a very high IR value (2.4-2.6) in paper II. As compared to paper I, the chances for a strong correlation was reduced in this paper, since a majority of the cell lines had similar IR values.

Survivin is an attractive marker since it is extensively overexpressed in tumour cells, and commonly low in normal tissue (Fukuda *et al.*, 2006). The results from paper II showed that the combination of survivin, Bax, Bcl-2, Bcl-X_L, COX-2, and the p53 Arg72Pro polymorphism correlated strongest to IR ($r=0.553$, $p<0.001$). Survivin was identified as a strong predictive factor for IR in paper I. The fact that it received significance on its own in paper II, among 42 cell lines, increases the probability for survivin being an accurate predictive marker for radioresponse. Survivin may be localised either in the nucleus or in the cytoplasm (Lippert *et al.*, 2007), and nuclear survivin has been shown to be an independent predictor of poor survival (Preuss *et al.*, 2008). This indicates that it could be advantageous to investigate the localisation of survivin even in terms of prediction of treatment response. A high expression of survivin is both in paper I and II correlated to radioresistance, which is in accordance with earlier findings (Capalbo *et al.*, 2007). However, others argue that overexpression of survivin gives a favourable outcome in oral squamous cell carcinomas receiving radiotherapy (Freier *et al.*, 2007). Survivin expression may be negatively regulated by normal p53 and cancer-associated p53 mutations may disrupt this regulation (Khan *et al.*, 2009, Mirza *et al.*, 2002). This is supported by the finding that transcriptional repression of survivin is induced by wild-type p53 (Mirza *et al.*, 2002, Xia *et al.*, 2006). Since numerous human cancers contain high frequencies of mutated p53 this may explain the striking overex-

pression of survivin in tumour cells (Ambrosini *et al.*, 1997), and the lack of survivin in adjacent normal tissue (Khan *et al.*, 2009). In four of the UT-SCC cell lines no p53 mutation was found. Three of these had a low overexpression of survivin, whereas one cell line had an intermediate overexpression. This could possibly indicate that normal p53 function suppresses the uncontrolled expression of survivin that can take place in cell lines where p53 is mutated.

Among the 42 investigated cell lines, 38 (90%) had one or more mutations in the p53 gene. P53 mutations are present in approximately 50% of all human cancers (Soussi *et al.*, 2007) and in HNSCC the prevalence varies from 30-70% (Blons *et al.*, 2003), leading us to believe that a selection takes place during the establishment of tumour cell lines that favours growth of p53-mutant tumour cells. P53 splice site/missense mutations improved the correlation to IR in paper I. When p53 mutations were analysed in paper II, they did not correlate significantly to IR, nor did they increase the correlation when combined with other factors. Yamazaki *et al* found that only specific mutations (DNA contact mutations) correlated to poor prognosis in HNSCC but the presence of any p53 mutation did not (Yamazaki *et al.*, 2003). This is contradictory to earlier findings that all p53 mutations were strongly associated with loco-regional treatment failure following radiotherapy (Alsner *et al.*, 2001).

The p53 Arg72Pro polymorphism has been correlated with poor therapeutic response and poor outcome in HNSCC (Kuroda *et al.*, 2007, Soussi *et al.*, 2007). We found that the p53 Arg72Pro polymorphism increased the correlation to IR when added to the proteins, indicating that functional p53 influences the IR.

Results paper III

The aim of paper III was to investigate the predisposition of the FGFR4 Gly388Arg polymorphism and its role in the development of HNSCC, and to examine if the FGFR4 Arg³⁸⁸ allele could be associated with resistance to chemo- and/or radiotherapy.

In samples from 110 tumour biopsies from the Linköping HNSCC tumour bank the FGFR4 Arg³⁸⁸ allele was found in 45% (49 out of 110) as compared to 58% (111 out of 192) in the 192 controls from the South-Eastern region of Sweden. We found that the Gly³⁸⁸ allele was associated with a significantly higher risk of developing cancer, OR 1.71 ($p=0.026$), suggesting the (wild-type) Gly³⁸⁸ allele as the risk allele considering predisposition for cancer. Males carrying this allele had a 2-fold risk, OR 2.0 ($p=0.031$), to develop HNSCC and it also gave a significantly higher risk to develop cancer in the oral cavity as opposed to the larynx, OR 2.49 ($p=0.002$).

We investigated the role of the Arg³⁸⁸ allele for radiation sensitivity in 39 cell lines and cisplatin sensitivity in 35 cell lines from HNSCC. There was no association between the Arg³⁸⁸ allele and radioresponse ($p=0.996$) but its presence tended to increase the sensitivity to cisplatin ($p=0.141$).

Furthermore, we analysed if the Arg³⁸⁸ allele in combination with high expression of the FGFR4 protein could influence the treatment sensitivity. Eight cell lines with different radio- and cisplatin sensitivity were analysed for FGFR4 protein expression (five cell lines with the Gly³⁸⁸ allele and three with Arg³⁸⁸), but no correlation between treatment sensitivity and expression levels of the FGFR4 protein was found in this small material. However, four cell lines showed 2-4 bands on the western blot that were not present in NOK. In order to explain the extra bands on the Western blot, the FGFR4 gene was sequenced and a novel mutation was found in six of the eight cell lines (UT-SCC-2,-9, 12A, -33, -34, -77). Four nucleotides into intron 13 there was an amino acid exchange altering a stop codon to a glutamine (T > C). The cell lines UT-SCC-9 and -33 both carried the novel mutation but had only weak FGFR4 protein bands in the western blot analyses. Therefore the FGFR4 mRNA expression was analysed in these two cell lines and in NOK, but mRNA was detected in comparable amounts in both cell lines and NOK, thus the solution to the weak bands could not be explained on mRNA level.

Discussion paper III

In this study we examined whether the FGFR4 Arg³⁸⁸ or Gly³⁸⁸ alleles affected the risk to develop HNSCC. Our results were unexpected and showed that the Gly³⁸⁸ allele was associated with a higher risk for HNSCC, OR 1.71 ($p=0.026$). In this study, the Arg³⁸⁸ allele seemed to protect against cancer development (as compared to the Gly³⁸⁸ allele). However, in a previous study it was demonstrated that if a cancer occurred in patients bearing the Arg³⁸⁸ allele, the progression of the disease was faster and the overall sur-

vival reduced as compared to patients with the FGFR4 Gly³⁸⁸ allele (Bange *et al.*, 2002, Streit *et al.*, 2004) suggesting that the Arg³⁸⁸ allele might predispose for development of distant metastasis, late recurrences, or second primary tumours (Bange *et al.*, 2002, da Costa Andrade *et al.*, 2007). FGFs are polypeptide growth factors that bind to FGFRs and trigger cascades of intracellular events leading to stimulation of cell growth by promotion of cell cycle progression and inhibition of cell death. These mechanisms are all potentially carcinogenic, since loss of regulation at any step can result in the driving of downstream processes that promote cell growth beyond control (Powers *et al.*, 2000). It can therefore be speculated that patients with the Arg³⁸⁸ allele maintain a balance in cell cycle and proliferation, while the patient with the Gly³⁸⁸ allele possibly escalates the downstream signalling and rushes cell cycle so that cell growth is driven beyond control and becomes carcinogenic.

Interestingly, we found that males carrying the Gly³⁸⁸ allele have a 2-fold risk of developing HNSCC as compared to women carrying the same allele. The incidence of HNSCC is three times higher among men than women and this difference has often been explained by the fact that men, at least historically, smoke to a greater extent than women. However, the results from this paper indicate that genetic variations such as FGFR4 polymorphisms, along with factors such as smoking are likely to contribute to the difference in incidence between the sexes. Furthermore, the Gly³⁸⁸ allele was associated with a greater risk of developing oral cavity cancer as opposed to cancer in the larynx. The underlying biological understanding of this finding is yet to be investigated.

In primary breast cancer the Arg³⁸⁸ allele is associated with a decreased survival, especially among the patients given chemotherapy (Thusbas *et al.*, 2006), indicating that the Arg³⁸⁸ allele could influence treatment sensitivity. According to the results in this paper, the Arg³⁸⁸ allele does not affect the IR ($p=0.996$). However, it may influence the cisplatin sensitivity since cell lines carrying the Arg³⁸⁸ allele tended to respond better to cisplatin treatment ($p=0.141$). Thus, the FGFR4 Arg³⁸⁸ allele may prove useful in combination with other factors for prediction of cisplatin response.

Streit *et al.* showed that FGFR4 expression alone had no impact on disease progression in HNSCC, but the overall survival time was significantly decreased in patients with the combination of the Arg³⁸⁸ allele and a high expression of FGFR4 protein (Streit *et al.*, 2004). Therefore, we wanted to investigate if this combination also played a role in treatment sensitivity; however, such a correlation could not be confirmed in our material.

A novel mutation in the FGFR4 gene was found in six out of eight investigated cell lines. The function of this mutation is unclear, and it remains unknown if a protein can at all be constructed from a gene containing this mutation. It could possibly result in a truncated protein with loss of exons 14-18, which are included among the exons re-

sponsible for intracellular signalling, indicating that such a protein is not likely to be functional. Intracellular signalling is likely to be inhibited; however, the biological effect of this mutation remains unclear.

Results paper IV

The aim of paper IV was to evaluate the possibility of using a panel of proteins and SNPs involved in apoptosis, growth control, and DNA-repair as predictive markers for cisplatin response.

In 39 cell lines, the ICS values were determined and given in surviving fraction of cells. The values varied from 0.00 to 1.00, with a median of 0.52. IR values were previously determined and survival data was fitted as a function of the radiation dose using a linear quadratic equation. The area under the curve (AUC) was obtained by numerical integration and varied between 1.4 and 2.6, with a median of 2.0. Pearson's correlation analysis did not show any association between IR and ICS and cell lines could be sensitive to one and resistant to the other treatment, sensitive to both, or resistant to both without a pattern.

The expression of EGFR, Hsp70, Bax, Bcl-2, Bcl-x_l, survivin, and COX-2 was determined using ELISA and correlated to cisplatin response. EGFR was the only protein that alone showed a significant correlation to ICS ($r=0.388$, $p=0.015$). The proteins were then arranged in the NNP system and the NNP sum for the different combinations of proteins was calculated for each cell line and correlated to the ICS. The combination of EGFR, Hsp70, Bax, and Bcl-2 yielded the strongest correlation to ICS among the proteins ($r=0.566$, $p<0.001$).

Since cisplatin forms DNA-adducts in exposed cells we hypothesised that SNPs within DNA-repair genes could affect the cellular response to this drug. Thus, the correlation between ICS and SNPs in four DNA-repair genes (XPC Ala499Val, XPD Lys751Gln, XRCC1 Gln399Arg, and XRCC3 Thr241Met) was investigated. All four SNPs tended to increase the cisplatin sensitivity as the median ICS of the group with at least one variant allele was lower than that of the homozygous wild-type group; however, none of these differences reached the level of significance. The FGFR4 Gly388Arg polymorphism also tended to influence the response to cisplatin. Nevertheless, when the XRCC3 and XPD polymorphisms were added to the combination of proteins with the strongest correlation to IR, both turned out to improve the correlation.

The combination of factors that correlated strongest to the ICS was extracted firstly with Pearson's correlation test and then verified by a multivariate analysis. The combination of EGFR, Hsp70, Bax, Bcl-2, XRCC3 Met²⁴¹, and XPD Gln⁷⁵¹ had the strongest correlation to ICS ($r=0.614$, $p<0.001$).

Since EGFR alone was found to significantly influence the ICS, we wanted to further evaluate its role for cisplatin resistance. The anti-EGFR antibody cetuximab was given alone or in combination with cisplatin to four cell lines with different levels of EGFR expression. Cisplatin alone triggered cell death only in the two cell lines with the lowest expression of EGFR (UT-SCC-12A, and -9). Cetuximab treatment resulted in cell

death in UT-SCC-9 and -24A, in which the EGFR is moderately (4-fold) and highly (19-fold) overexpressed, respectively. Three out of four cell lines tested responded only to one of the treatments. Only in UT-SCC-9 an additive effect between cisplatin and cetuximab was observed.

Discussion paper IV

In this study we evaluated the possibility of using a panel of proteins and SNPs as predictive markers for cisplatin response. Cisplatin is the most effective platinum-based chemotherapy and is often used in combination with radiotherapy in the treatment of locally advanced HNSCC. However, as cisplatin causes severe neuro-, nefro-, and ototoxic side-effects and since only about 25% of the patients respond effectively to the treatment, prediction of ICS would be of utmost importance. If patients resistant to cisplatin treatment could be identified, they could be treated with surgery, radiation, and/or a molecularly targeted drug like cetuximab and be spared the side-effects of cisplatin.

It has been hypothesised that a patient sensitive to radiotherapy would also be cisplatin sensitive. Paper IV proves this theory wrong. Statistical analysis clearly shows that the IR and ICS do not correlate ($r=0.138$, $p=0.401$), implying that patients may benefit from one treatment but not the other, both treatments or none of them. Furthermore, we see that the panel of predictive markers correlating to IR in paper II (survivin, Bax, Bcl-2, Bcl-X_L, COX-2, and the p53 Arg72Pro polymorphism) and the combination that strongest predict ICS in this paper (EGFR, Hsp70, Bax, Bcl-2, XRCC3 Thr241Met, and XPD Lys751Gln) is not in accordance. Only Bax and Bcl-2 are included in both panels, however, these two seem to be of less importance than stronger predictors like EGFR and survivin.

In papers I (n=9), and IV (n=39), EGFR had relevance for radiosensitivity and cisplatin sensitivity, respectively. This growth factor receptor has several functions within the cell including DNA repair. Cisplatin causes DNA breaks that normally lead to apoptosis. Furthermore, it induces translocation of EGFR to the nucleus, where it interacts with the DNA-repair enzyme DNA-dependent protein kinase and stimulates repair of the DNA strand (Hsu *et al.*, 2009). This mechanism can lead to an increased cisplatin resistance (Chen *et al.*, 2007) as DNA is more efficiently repaired and the cell can avoid apoptosis. Likewise, cisplatin resistance of the cell line UT-SCC-26A was overcome by stimulation of EGFR (Mandic *et al.*, 2009). EGFR, which is reported to be overexpressed in 55-100% of HNSCC, was upregulated in 57% of our cell lines.

Bax and Hsp70 were the two proteins with highest influence on ICS following EGFR. This was partly confirmed by Miyazaki *et al.* who showed that cells with a high expression of Hsp70 responded poorly to both chemo-radiotherapy and radiotherapy as a single treatment. The Bax expression; however, was not related to the efficacy of

therapy (Miyazaki et al., 2005). Hsp27 have been shown to confer chemoresistance but does not show any protective effect against radiation (Lee et al., 2007) and the function of Hsp27 and Hsp70 is often considered similar.

The presence of polymorphic variants of the DNA repair genes XRCC1, and XPD have been reported as prognostic factors for cisplatin treatment in HNSCC (Quintela-Fandino *et al.*, 2006). In line with this, SNPs within the XPD and XRCC3 genes were linked to cisplatin response in esophagogastric cancer (Font *et al.*, 2008). In this study, all the investigated SNPs in DNA-repair genes tended to increase cisplatin sensitivity. When combined with the proteins the XRCC3 Thr241Met and XPD Lys751Gln were included among the factors that increased the correlation to ICS. Thus indicating that those two had a greater predictive value for ICS than the other investigated SNPs.

GENERAL DISCUSSION

Studies on tumour cell lines

In this thesis cell lines were used in order to understand how tumour cells with different characteristics respond to various treatments. The foremost advantage with cell line experiments is that there is an unlimited supply of renewable cells for experiments. It is often far more difficult to establish tumour cell lines from biopsies as compared to normal cells (O'Hare, 1991). Furthermore, it is commonly the most aggressive clones within the tumour biopsy that are likely to survive the first fatal steps of cell culturing (Hsu, 1999). It can, however, be argued that it is these clones that are of greatest interest in studies on prediction of treatment resistance, since they are more likely to survive the given treatment. Interestingly, of the cell lines studied in this thesis 90% had p53 mutations, while HNSCC generally is reported to harbour p53 mutations in 30-70% of the cases (Blons *et al.*, 2003). The higher frequency of p53 mutations among our cell lines indicate that cells carrying mutated p53 might have a growth advantage in cell culture.

Cell lines are generally highly representative of the cancers from which they are derived. In a study on 127 cancer cell lines producing tumours in nude mice after subcutaneous injection, the histopathology of the tumours correlated with that of the original tumours in all cases (Fogh *et al.*, 1977). Even after lengthy times of cell culturing breast and non-small cell lung cancer cultures retained the properties of their parental tumours (Wistuba *et al.*, 1998, Wistuba *et al.*, 1999). For head and neck cancer we know that oral squamous cell carcinoma cell lines had the same IR values after numerous passages (personal communication, Professor R. Grenman). A common pitfall with cell line experiments is that variant clones may develop with a phenotype and/or genotype differing from the predominant population. These clones can be faster growing or better adapted to the culture conditions and are thereby likely to be selected (Masters, 2000). Therefore, all experiments in this thesis were performed on cell lines in relatively low passages, 10-25.

HNSCC cell lines are derived from tumours of several different anatomical sites and possibly should be studied organized on the basis of their site of origin. In paper III we observed that the FGFR4 Gly³⁸⁸ allele was associated with a significantly higher risk to develop cancer in the oral cavity (OR 2.49, $p=0.0002$) while no such correlation was seen in tumours from the larynx, indicating that genetic aberrances indeed can have diverse effects depending on the anatomical site of the tumour.

One possible objection towards the use of cell lines is the lack of the potential influence from cells of the tumour microenvironment. Increasing amounts of data sug-

gest that for example tumour-associated fibroblasts could modulate the response to cancer therapy (Hwang *et al.*, 2008, Shekhar *et al.*, 2007). However, the lack of environmental influences is also beneficial in the respect that different parameters can be more securely controlled. Moreover, the response to different treatments like radiation, chemotherapeutic agents, and molecularly targeted drugs can be evaluated in the tumour cells without the influence from surrounding stromal cells.

The NNP system

The World Health Organisation predicts a continuing worldwide increase in the incidence of oral cancer, extending into the next several decades (Bettendorf *et al.*, 2004). With new and more specific treatments like molecularly targeted therapy, treatment will be increasingly expensive. Resistance to treatment, treatment morbidity, and local recurrences are significant problems during and after therapy, highlighting the need for improved and individualized treatment. For this reason, it is necessary to have a panel of predictive markers in order to give the most effective treatment, tailor-made for the special characteristics of every tumour (Hanash *et al.*, 2008, Thomas *et al.*, 2005). We have evaluated the usefulness of a panel of factors in predicting the response to radio- and cisplatin treatment. We believe that the NNP system can be a helpful tool in the optimization and individualization of the choice of treatment for HNSCC patients. A weakness of the NNP system could be that neither the complex relationship between factors nor the biological significance of each included factor is taken into account. The system has in this thesis been tested for IR and ICS, but the intention with the NNP system was that it should be applicable to any treatment and predictive markers of all types, including those not yet known.

One of the major advantages with the NNP system is the possibility to combine multiple predictive markers. This is something that has been asked for although no previous studies have displayed means for a study design addressing this. However, in 2009, Van den Broek *et al.* published an interesting paper where sets of prognostic factors were combined using first proportional hazard analysis to investigate associations between each individual marker and outcome. In addition the global test by Goeman was used (van den Broek *et al.*, 2009). Goeman introduced the global test as a tool for analysis of microarray data (Goeman *et al.*, 2004), however, van den Broek used it when testing 18 biomarkers, or selected combinations, for an overall association with local control. Related variables were grouped in pathways according to; cell cycle control, apoptosis, hypoxia, and chemotherapy sensitivity. Thereafter each group was tested for association with local control. We believe, however that cell cycle control, apoptosis and treatment sensitivity are closely related mechanisms, where factors involved in one system can also trigger events in others. Therefore, the NNP method

could be a more useful method, since each factor is correlated independently, and then in all possible combinations, to the treatment sensitivity analysed.

Clinical implication

Using the NNP system we try to identify factors with strong predictive possibility and exclude factors with a weak correlation to treatment response. Our goal is to find a panel consisting of strong markers that can easily be transferred to the clinical reality. A limited number of reliable markers are important since we need to minimize the analyses required due to costs and shortage of time until the treatment should begin.

A problem with performing the investigations on tumour material as opposed to cell lines is the mixture of normal cells and tumour cells within a biopsy. An area within the biopsy containing a majority of tumour cells would have to be chosen and analysed for protein expressions. The analyses for SNPs and mutations could be performed within the time limit, however with increasing costs since each sample would need individual analyses, as soon as the patient arrives in the ear-nose and throat department, as opposed to a set of samples tested together during research.

The response to radiotherapy will be difficult to evaluate in the clinical setting until more standardized protocols for this evaluation has been introduced. Today a subjective evaluation is made by the surgeon at the first clinical control after irradiation and an investigation in the charts indicate that "good response" to radiation is the most specific term given. Evaluating the response to cisplatin will be even harder since a limited number of HNSCC patients in Linköping have received cisplatin treatment, other than for palliation so far. Linköping would need to collaborate with other head and neck cancer centres in order to increase the material size. To enable standardized treatment evaluation, radiology possibly including PET/CT would probably be needed. Combined treatments including chemoirradiation is now indicated for advanced HNSCC (Pignon et al., 2007, Vermorken et al., 2008), requiring even more complex systems for evaluation to separate the effects between the two treatments, if at all possible. New combinations of chemotherapy and cetuximab are also evaluated and show slightly prolonged survival times (Vermorken et al., 2008). These problems motivate further pre-clinical investigations until the cellular mechanisms are better understood and response rates more securely can be predicted.

The introduction of novel molecularly targeted therapies and combined treatment regimens escalates the costs for cancer management. One of the major advantages of the recently introduced treatments including cetuximab and survivin inhibitors is that side-effects seem to be less severe than those associated with conventional treatment regimens. If the expected response to different treatments was possible to predict for each patient, they could quickly receive the most promising therapy and be spared the side-effects of ineffective treatments. Until reliable predictive markers are found, the

greater number of different treatments given the greater the chance that a patient responds to one of the treatments. That is why contributing to finding panels of markers that can predict treatment response and models to enable combination of factors is of such vital importance and will continue until individualised treatment regimens will be a part of the every-day life of clinicians treating head and neck cancer patients.

CONCLUSIONS

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

1. It is crucial to combine multiple factors to securely predict treatment response.
2. The NNP system is a valuable tool for the simultaneous evaluation of multiple factors on protein and gene level.
3. Survivin is a strong predictive marker for radioresponse.
4. EGFR is a strong predictive marker for response to cisplatin treatment.
5. Combinations of predictive markers increase the possibility to predict treatment sensitivity.
6. The FGFR4 Gly³⁸⁸ allele is a risk factor for the development of HNSCC. Males carrying this SNP had a 2-fold increased risk to develop cancer. The risk of getting a tumour in the oral cavity was increased as compared to the larynx.

FUTURE

The next study will be performed on tumour biopsies from the Linköping material, and the protein expression will be analysed using immunohistochemistry and thereafter tested for correlation to treatment response in two groups of patients (responders and non-responders to radiotherapy). A panel of factors on both protein and gene level will be investigated and combined in the NNP model. We will continue to use the NNP model for the combining of predictive markers until a better system is developed. The search for new predictive markers will go on and the first marker to be investigated by us is the correlation between WRAP 53 expression and IR in the Linköping material. The continuation of this thesis will focus on the task of finding strong predictive markers for therapy response, starting with WRAP53. Other factors of interest are HPV-positivity/p16, and fibronectin 1 and these could be included in the panel investigated in the tumour biopsies.

SUMMARY IN SWEDISH

I Sverige insjuknar varje år ungefär 850 personer i skivepitelcancer i huvud- och halsområdet. Denna typ av cancer är den sjätte vanligaste i västvärlden, medan den i vissa utvecklingsländer är den näst vanligaste cancerformen. Två tredjedelar av dem som drabbas är män och ännu har inga ärftliga riskfaktorer upptäckts. Istället är det riskfaktorer så som rökning, gärna i kombination med hög alkoholkonsumtion, och infektioner med humant papillomvirus, som ger upphov till tumörer.

Trots förbättrade operationsmetoder och intensifierad onkologisk behandling, har inte femårsöverlevnaden förbättrats nämnvärt under de senaste tjugo åren. För att möjliggöra förbättring av både behandling och prognos för patienterna med huvud- och halscancer, är det viktigt att studera vad som gör att vissa patienter svarar bra på en behandling som en annan patient inte har någon nytta av.

Det är välkänt att tumörceller över- eller underuttrycker proteiner och har genetiska förändringar, som ger tumören tillväxtfördelar jämfört med normala celler. Genom att mäta flera av dessa förändrade faktorer, tror vi att det finns en möjlighet att också kunna förutspå behandlingssäkerhet. Studier har så här långt visat att det inte räcker att titta på en enskild faktor för att säkert kunna förutspå behandlingssvar. Därför har vi undersökt många olika proteiners uttryck och genetiska förändringar och kombinerat dem, för att försöka förutspå hur tumörer i huvud- och halsområdet kommer att svara på strålbehandling och cellgiftet cisplatin. För vardera av dessa två behandlingar har vi tagit fram en kombination av biomarkörer som korrelerar till känsligheten för respektive behandling. Vi har också undersökt vilka av dessa faktorer som enskilt har en stark koppling till behandlingssäkerheten.

I ett arbete studerades en specifik genetisk förändring i den så kallade FGFR4 genen. Det visade sig att den var kopplad till en ökad risk att utveckla huvud- och halscancer. Risken var störst för tumörutveckling i munhålan, samt dubbelt så hög för män, jämfört med för kvinnor.

Sammanfattningsvis var målet med denna avhandling att ta fram faktorer, som i kombination, kan ge svar på hur känslig en tumör kommer att vara för olika behandlingar. På sikt hoppas vi kunna bidra till att behandlingen inom huvud- och halscancer skraddarsys för varje enskild patient, så att uteslutande effektiv behandling ges, med minimal biverkningsprofil.

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