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# **On Predictive Factors of Treatment Response in Head and Neck Squamous Cell Carcinoma**

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## **ABSTRACT**

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer and yearly include 500 000 new cases worldwide. The outcomes for these patients have not been significantly improved over the last decades and the five year survival is still around 50 %. Establishing predictive markers of treatment response will have great impact on the clinical management of this disease.

The aim of this thesis was to elucidate markers of intrinsic response to radiotherapy and cisplatin. Combining expression patterns of 14 proteins and identifying mutations in the p53 gene, we were able to incorporate both protein and genetic changes to create a predictive model termed Number of Negative Points (NNP). We used the NNP model to statistically calculate the combination of factors that had the best correlation to intrinsic radiosensitivity (IR). We established that a panel of three markers, epidermal growth factor receptor (EGFR), survivin and splice site/missense mutations of p53, had the best correlation to IR ( $R=0.990$ ,  $p<0.0001$ ).

We also conducted gene expression analysis to investigate what genes and gene ontologies that are different between cell lines with varying IR. Furthermore, we wanted to identify key regulator genes with central positions of molecular networks, which were generated from the transcripts included in the deregulated gene ontologies. A transcriptional profile of 28 key regulator genes was generated. Immunoblot analysis supported deregulation at the protein level of three markers implicated from the transcriptional profile. We propose that these proteins, notch1, thrombospondin 1, and pai-1 are predictive markers of IR.

Finally, on a subset of cell lines with sensitivity or resistance to cisplatin, we performed gene expression analysis. Markers of intrinsic cisplatin sensitivity (ICS) such as gene ontologies and key regulators of molecular networks were proposed and five genes, APOE, CTNNB1, MMP7, MMP13, and THBS1 were selected for further analysis. Quantitative polymerase chain reaction (qPCR) analysis of these genes in 25 cell lines established that MMP7 ( $p=0.0013$ ) and MMP13 ( $p=0.058$ ) are possible predictive markers of ICS.

The markers of IR and ICS presented here could, if confirmed in a clinical setting, guide clinicians in the choice of treatment and thus give a more individualized and effective therapy for patients with HNSCC.



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## **LIST OF PAPERS**

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

- I. Lovisa Farnebo, **Fredrik Jerhammar**, Linda Vainikka, Reidar Grénman, Lena Norberg-Spaak and Karin Roberg. Number of Negative Points: A novel method for predicting radiosensitivity in head and neck tumor cell lines. *Oncology Reports* 2008; 20(2) 453-61
- II. **Fredrik Jerhammar**, Rebecca Ceder, Reidar Grénman, Roland C. Grafström and Karin Roberg. Identification of key regulator genes linked to radioresistance in head and neck squamous cell carcinoma by bioinformatic processing of transcript data. *Submitted*
- III. Anna Ansell, **Fredrik Jerhammar**, Rebecca Ceder, Reidar Grénman and Karin Roberg. Matrix metalloprotease-7 and -13 predict response to cisplatin in head and neck cancer. *Manuscript*



## **INTRODUCTION**

### **HEAD AND NECK CANCER**

Head and neck cancer comprise 5% of all cancer cases worldwide, which represents 500 000 new cases every year(1). The equivalent numbers for Sweden was 1,7 % of total cancer cases for the year 2006, which corresponded to 843 new cases of head and neck cancer (2). Originating in the mucosal lining of the upper respiratory and digestive tracts, 95% of the cancers in this region are of squamous cell origin. The remainder mainly consists of salivary gland malignancies.

The most important risk factors for development of head and neck squamous cell carcinoma (HNSCC) are tobacco and alcohol consumption (3). Human papillomavirus (HPV) infection is also an established risk enhancer (4).

### **TREATMENT OF HNSCC**

The prognosis is good for a patient diagnosed with early stage disease (stage I or II), for which numbers of favorable outcome of up to 90% is available in the literature (3). These patients are treated with surgery and/or radiotherapy, depending on the site of the primary tumor. However, two thirds of HNSCC patients are diagnosed with locally advanced disease (stage III and IV), for which the survival is significantly lower. The treatment options in the advanced stages are surgery, radiotherapy, chemotherapy, or concurrent chemo-radiotherapy.

#### *Radiotherapy*

Radiotherapy remains a core treatment for HNSCC. Efforts are made to enhance the dose delivery to the tumor and to reduce the toxic effects on normal tissue by novel approaches such as intensity modulated radiotherapy (IMRT) and altered fractionation schedules. The IMRT technique renders a possibility to adjust the radiation dose according to the three-dimensional structure of the tumor, which ultimately minimizes the exposure to surrounding normal tissue. Such

therapy has recently been shown equally effective as conventional radiotherapy, but with significantly less late toxicities (5).

Studies of altered fractionation schedules, such as accelerated fractionation and hyperfractionation, have been frequently reported over the last decade. Bourhis *et al.* conducted a meta-analysis with the purpose of comparing accelerated fractionation to hyperfractionation in which 15 trials were included. They conclude that altered fractionation schedules improve survival of HNSCC patients, and that hyperfractionation was the best type of altered radiotherapy (6).

### *Chemotherapeutic agents*

A panel of chemotherapeutic agents including taxanes, anti-metabolites and platinum compounds are utilized in the clinic. The platinum compound cisplatin is regarded as a standard agent, and is often used in combination with radiation or other compounds. Induction therapy, where often cisplatin is combined with 5-fluorouracil (platinum + fluorouracil, PF) and sometimes also docetaxel (taxane + PF, TPF), has gathered increased interest (7).

### *Chemoradiotherapy*

In many countries the standard treatment regimen for unresectable tumors is chemoradiotherapy, because of the higher survival numbers compared to radiation alone (8). A large meta-analysis by Pignon *et al.* establishes that chemoradiotherapy is slightly superior in terms of absolute survival (8% higher at 5 years from diagnosis), although associated with an increased toxicity (9). Mucositis and other complications are common in concurrent chemoradiotherapy which is widely used but, due to the high toxicity, somewhat debated.

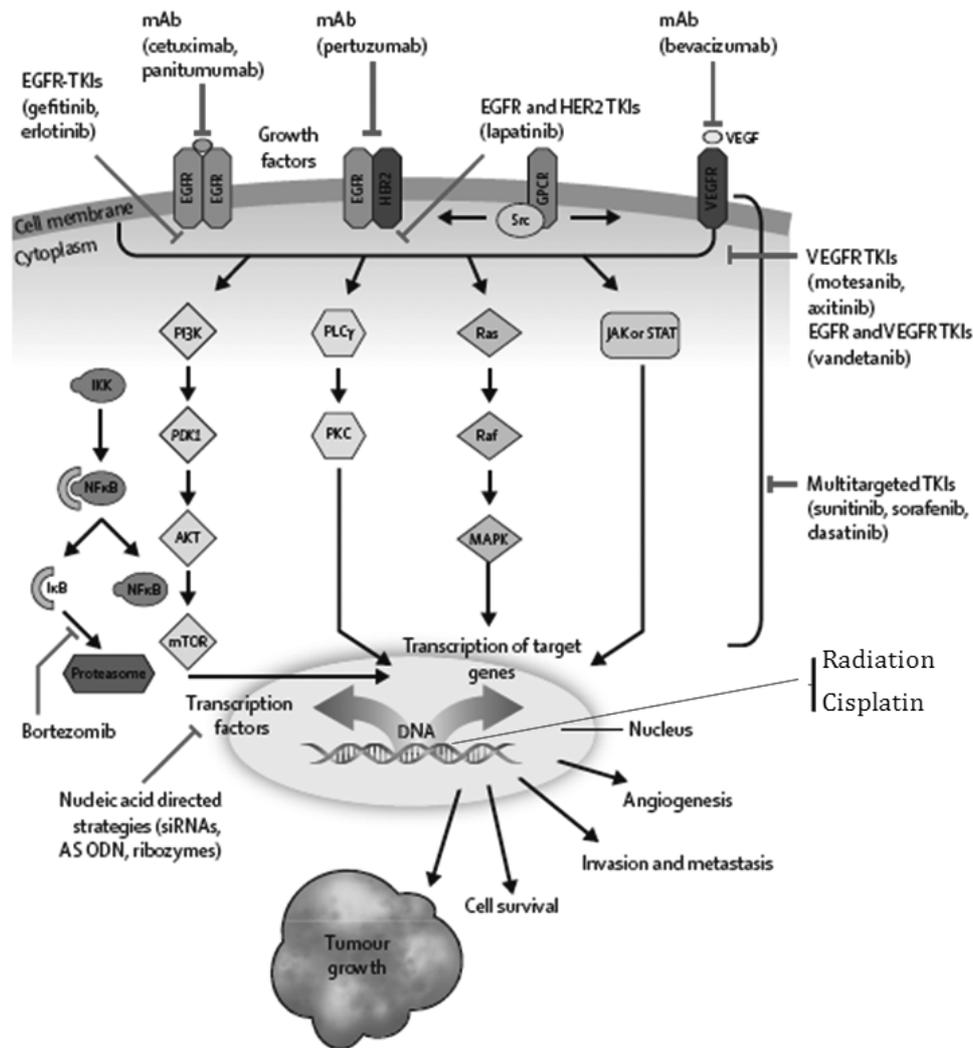
Induction therapy in combination with chemoradiotherapy has also been studied recently. In a setting with 501 patients with advanced HNSCC, TPF was found superior to PF when both induction therapies were combined with chemoradiotherapy (7).

## *Novel therapies*

Targeted therapy is an interesting and promising field in cancer therapeutics. The drug industry has launched an arsenal of compounds, mostly targeting growth factor receptors or their downstream effector molecules. Figure 1 summarizes some of these compounds and the pathways they intervene.

One agent that has shown clinical potential in the treatment of HNSCC is Cetuximab (C225, Erbitux®), which is a monoclonal antibody directed towards the epidermal growth factor receptor (EGFR). Its activity at the ligand binding site of the receptor inhibits the downstream signaling and ultimately hampers the EGFR-coupled gene expression.

Clinical studies have implied the usefulness of cetuximab in different combinatory regimes. In a randomized multicentre study conducted by Bonner *et al.* it was demonstrated that there is an increase in overall survival after treatment with cetuximab in combination with radiotherapy as compared to radiotherapy alone (49.0 months compared to 29.3 months) (10). Furthermore, it was recently shown that Cetuximab could also improve overall survival when combined with PF compared to PF alone (10.1 months to 7.4 months) (11).



**Figure 1.** Schematic overview of molecular signaling implemented in HNSCC and pathway intervening treatment options. Modified from Argiris et al. *Lancet* 2008;371(9625):1695-709.

Despite the different treatment regimens available, about 50 % of patients with HNSCC experience local or distant relapses. These numbers have remained unchanged over the last decades which clearly motivate extended research of the pathological changes underlying this disease. The potential of individualized treatment is widely anticipated in the medical research community. This, however, requires reliable predictive markers of treatment response which is currently not available.

## PREDICTIVE AND PROGNOSTIC MARKERS

A prognostic marker is a biological trait that can be used to estimate the outcome of a particular disease. A predictive marker, on the other hand, is a characteristic which relates to the treatment response. Clinical diagnostic evaluation of cancer progression includes histologic appearance, tumor grading, lymph node involvement and presence of distant metastasis. These are valuable as prognostic indicators but are of limited importance in prediction of treatment responses (12).

Numerous prognostic HNSCC markers are available in the literature, but few have a validated clinical value. As previously described, advanced tumor stage and nodal involvement are indicative of low survival. HPV infection increases the risk of developing HNSCC, but is also a positive factor for favorable outcome (13).

The establishment of prognostic markers is important for the understanding of tumor progression and identification of new therapeutic markers (14). Such markers are foreseen and extensively sought after.

### PREDICTIVE MARKERS OF TREATMENT RESPONSE

The course of cancer progression is complex and involves multiple deregulated biological processes such as growth control, apoptosis and angiogenesis. The growing arsenal of therapeutic regimes and compounds is intriguing. However, in order to reach significantly elevated survival numbers, the establishment of predictive markers of treatment response is crucial.

#### *Radiotherapy*

Proteins that control apoptotic cell death have impact on the cellular response to radiation. The balance between anti- and pro-apoptotic members of the Bcl-2 family regulates cellular fate, and altered expression has been suggested to influence cellular radioresponse (15-17). Anti-apoptotic proteins (such as Bcl-2 and Bcl-XL) and pro-apoptotic markers (e.g. Bad, Bak, Bax, and PUMA) have implied a possible predictive significance in need of further evaluation (18).

Inhibitors of apoptosis (IAPs) are also crucial regulators of apoptosis. Survivin is an IAP which is usually unseen in terminally differentiated adult tissue, but is detected in many human cancers (19). Overexpression of survivin has been linked to cellular evasion of apoptosis following irradiation (20). Factors that are involved in radiation induced DNA-damage response have been proposed as potential markers (e.g. p53, Ku70, XRCC3, XRCC5, DNA-PK, ATM, and RAD51) but studies have so far been unable to validate their clinical importance (21, 22). Another important biological factor which influences the response to radiotherapy is hypoxia. The generation of reactive free radicals that damage DNA is enhanced by the presence of oxygen. Radiation is therefore more effective in patients with well-oxygenated tumors (21), which is also exemplified by decreased overall survival in patients that overexpress the hypoxia marker hypoxia inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) (23). Tumor cell proliferation, or rather repopulation between treatment occasions, seems to be another factor that hampers local control after radiotherapy (21). The relative advantages of altered fractionation schedules might be explained by impeded tumor cell repopulation (6).

### *Cisplatin*

Cisplatin functions through the forming of DNA-adducts, primarily by intrastrand crosslinking, and involves pathways such as DNA repair, cell cycle arrest, and apoptosis, which all converge on p53 signaling (24, 25). Treatment response does not correlate to p53 expression in HNSCC (22). However, reduced cisplatin sensitivity of head and neck squamous cell carcinoma correlates with mutations affecting the nuclear localization of p53 (26), indicating an importance for functional p53 in the signaling cascade in response to cisplatin treatment. Another interesting molecule is the excision repair cross-complementation group1 protein (ERCC1), which has been proposed as a predictive marker of cisplatin response. It is capable of removing platinum-containing DNA adducts and a low expression of ERCC1, detected by immunohistochemistry, positively influences the outcome of therapy (27, 28). Similarly, presence of single nucleotide polymorphisms (SNPs) of the DNA-repair genes XRCC1, XPD, and

ERCC1 could predict a better response to cisplatin (29). These results have yet to be validated in a larger material.

### *Cetuximab*

Studies have shown clinical achievement of cetuximab, but lack of molecular markers of treatment response has limited the advantages. Recently Karapetis *et al.* established mutations of the k-ras gene as a predictive marker for cetuximab failure in colorectal cancer (30). However, k-ras mutations are rarely seen in HNSCC (31).

### GENE EXPRESSION ANALYSES IN HNSCC

Microarray technology has opened the possibility of simultaneous gene expression analysis of thousands of genes. Tests that predict clinical outcome on the basis of gene expression are likely to positively affect cancer treatment and increase the use of a personalized medicine approach (32). Questions have been raised regarding the reproducibility of microarray results, and there are examples of gene expression signatures that, even though validated in independent patient cohorts, show relative little overlap of genes (33). It has also been stated that differential gene expression on transcript level matches protein abundance to only about 40% (34). However, deregulation of gene product levels correspond to a higher extent regarding functional categories, albeit not for individual genes (35).

The search for predictive markers of treatment response is based on comparison between sensitive and resistant samples, where high-throughput analyses promises upcoming accomplishments. However, gene expression studies of head and neck carcinomas have primarily focused on identifying signatures of cancer progression (36-39), representing interesting aims although distinct from predictive marker evaluation.

Extensive data are emerging in the field of predictive chemotherapeutic markers. Several studies propose gene expression signatures that can guide the use of chemotherapeutic drugs in breast cancer management (40, 41). Takashima *et al.* established a gene list of 38 markers differently expressed in esophageal cancer cell lines

resistant to cisplatin (42), involving functional categories like cell cycle and signal transduction. Radiotherapy related transcriptomic analyses of HNSCC have mainly compared pre- and posttreatment gene-expression profiles (43-45), detecting deregulated functional categories such as cell cycle and cellular growth and proliferation (43). Arguably, basal gene expression patterns observed in the cell lines before treatment is likely to serve better as a predictor of intrinsic treatment resistance (44).

## **AIM**

Using a panel of HNSCC cell lines with quantitated intrinsic cisplatin and radiosensitivity, the aim of this thesis was to establish predictive markers of intrinsic radiosensitivity (IR) and intrinsic cisplatin sensitivity (ICS). Furthermore, we also wanted to:

- Establish a predictive model that includes both protein expression and gene mutations in a correlation analysis to IR.
- Conduct unsupervised gene expression analyses in order to identify functional groups and key regulators of molecular networks that predict IR and ICS.
- Verify single markers suggested as key regulators and their association to IR and ICS.

## **MATERIALS AND METHODS**

### **CELL LINES**

The HNSCC cell lines used in the papers of this thesis are all from the UTSCC-series established by Professor Reidar Grénman, University of Turku, Finland (46, 47). From a total of 42 cell lines we have, for each study, chosen a number of cell lines to represent different parts of treatment sensitivities. Table I summarizes the characteristics of the cell lines used. The cell lines used for quantitative PCR analysis are summarized in table I of paper III.

#### **PAPER I**

Nine HNSCC cell lines, representing different parts of the *in vitro* radiosensitivity spectrum, were selected for this study. Normal oral human keratinocytes (NOK), cultured as previously described (48), were also included in the study.

#### **PAPER II**

In this study five UTSCC cell lines were selected for microarray analysis, to represent different parts of the *in vitro* radiosensitivity spectrum. Two of the cell lines were highly resistant, two displayed intermediate resistance, and one was sensitive.

#### **PAPER III**

Three cell lines from the UTSCC-series were selected on the basis of their intrinsic cisplatin sensitivity (ICS). Two of the cell lines were resistant to cisplatin and one was extremely sensitive. The ICS of 35 cell lines was previously established in these cell lines by a clonogenic assay (49).

**Table I.** Characteristics of the cell lines used in this thesis. 1. Radiation sensitivity given in terms of mean inactivation dose (Previously published by Pekkola-Heino *et al* (46) and Erjala *et al* (50). (AUC=area under curve, IR= Intrinsic Radiosensitivity, ICS= Intrinsic Cisplatin Sensitivity)

Cell line	AUC <sup>1</sup> (IR)	t	ICS (% survival)	Used in Paper
UT-SCC-24A	2.6±0.3	100		I, II, III
UT-SCC-77	2.5±0.2	34		I, II
UT-SCC-33	2.3±0.2	70		I, II
UT-SCC-34	2.1±0.1	75		I
UT-SCC-12A	2.1±0.1	0		I, II, III
UT-SCC-2	1.8±0.2	100		I, III
UT-SCC-19A	1.7±0.1	35		I
UT-SCC-23	1.6±0.1	7		I
UT-SCC-9	1.4±0.1	89		I, II

## ASSESSMENT OF INTRINSIC RADIOSENSITIVITY

The Intrinsic Radiosensitivity (IR) of 42 HNSCC cell lines of the UT-SCC series was previously determined using a 96-well plate clonogenic assay (46, 47). Survival data as a function of radiation dose were fitted by a linear quadric equation, and the area under curve (AUC) was obtained by numerical integration (51). For each cell line a minimum of three experiments were performed. The average IR in a large panel of HNSCC cell lines was 1.9 for head and neck cancer of all sites (N=37) (46).

## ASSESSMENT OF CISPLATIN SENSITIVITY

The cytotoxic effect of cisplatin was determined in 35 HNSCC cell lines by a clonogenic assay (49). Tumor cells were seeded into six-well plates at concentrations of 200-400 cells/cm<sup>2</sup> depending on the plating efficiency of each cell line. After 24 h cells were exposed to 1µg/ml cisplatin for 1 h and incubated for another nine days before fixation in 4% formalin. Cells were then stained with 2% Giemsa and colonies containing 32 cells or more were counted. The cloning efficiency of untreated cells (control) was set to 100%, and the cloning efficiency of treated cells was expressed as a percentage of the control

value. In each experiment all cell lines were exposed in triplicate and the mean value was used for statistical analysis.

## **WESTERN BLOT**

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

In Paper II, western blot was used to evaluate the protein level expression values implied on RNA level in the microarray analysis.

## **NUMBER OF NEGATIVE POINTS**

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

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

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

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

sequencing was then performed in order to isolate the specific nucleotide sequence.

## **MICROARRAY**

Gene expression analysis on the microarray platform enables simultaneous determination of transcript levels of many thousands of genes. The particular chip used in Papers II and III (Affymetrix Human Genome U133 Plus 2.0 chip) analyses the expression of 38 500 genes. The vast amount of data that is extracted from these analyses requires appropriate statistical and bioinformatic processing, in order to yield useful results. Both papers have a similar setup, although the treatment sensitivity addressed is radiation in Paper II and cisplatin in Paper III. The overall ambition was to use an unsupervised approach to reveal biomarkers of treatment response. By comparing the differently expressed transcripts that are shared between two resistant cell lines compared to a reference cell line we can, to a higher extent, isolate the phenotype of resistance to the treatment and examine the pathways that are mutually changed. Subsequent bioinformatic analysis is likely to reveal more information on pathways regulating treatment resistance.

## **QUANTITATIVE REAL-TIME PCR (qPCR)**

In paper III, the microarray analysis implied several biomarkers that were differently expressed in the resistant cell lines compared to the sensitive cell line. Five markers were chosen for verification studies. Firstly, we wanted to verify the relative expression differences implied from the microarray analysis in the three cell lines. Secondly, we wanted to assess these markers in independent tumor material, i.e. a larger panel of cell lines. By qPCR analysis, the relative expression values of mRNAs are detected by calculation of the  $\Delta C_t$  value which corresponds to the cycle number that the PCR product reaches a predefined threshold number.

## BIOINFORMATICS

### GENE ONTOLOGY TREE MACHINE

The Gene Ontology Consortium<sup>1</sup> started out as a collaborative project between three model organism databases (FlyBase, Saccharomyces Genome Database and Mouse Genome Database). The project aims to provide a consistent vocabulary to explain the function of gene products from three main categories, that is, biological process, molecular function and cellular component. We have in Papers II and III compared resistant cell lines to reference cell lines and analyzed the differences in gene expression for enrichment of gene ontology categories. To carry out this analysis we used Gene Ontology Tree Machine (GOTM)<sup>2</sup>, which is a web-based statistical hypergeometric test applied for enrichment analysis of Gene Ontology (GO) categories.

### INGENUITY PATHWAY ANALYSIS (IPA)

The IPA knowledge base<sup>3</sup> contains roughly 2 million peer-reviewed articles from the scientific literature and is manually curated by PhD scientists. By using the IPA-tool to construct molecular networks we wanted to find key players of the pathways identified in the gene ontology analysis. Fisher's Exact test was used for ranking and significance analysis of the focus genes in the network. Selection of key regulators in the networks was based on interactions with at least three altered transcripts (52).

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<sup>1</sup> <http://www.geneontology.org>

<sup>2</sup> <http://bioinfo.vanderbilt.edu/gotm>

<sup>3</sup> <http://www.ingenuity.com>

## **RESULTS AND DISCUSSION**

### **PAPER I**

A requirement for increasing favorable outcomes of cancer patients is to discover markers of treatment response. Many markers have been implied in preclinical studies, but few have shown a clear cut clinical importance (53). Markers of proteins and genes have been studied but have failed to make their way into the everyday practice of HNSCC management.

This study aimed to combine markers that were previously suggested as important markers in preclinical studies and correlate them to intrinsic radiosensitivity in nine HNSCC cell lines, whose origin and IR are summarized in table I of paper I. 14 proteins involved in growth control and/or apoptosis along with p53 mutations of three different kinds were analyzed. Using western blot densitometric expression values adjusted to actin expression and standardized to NOK, we could assess the importance of each marker for the IR of the cell lines. The proteins analyzed were; Bcl-2, Bcl-X<sub>L</sub>, Bax, Bad, Bak, PUMA, Cyclin D1, Smad4, Hsp-70, EGFR, Survivin, COX-2, p53, and Mdm2. None of the proteins could alone show a significant correlation to IR.

EGFR is commonly overexpressed in HNSCC and is highly anticipated as a therapeutic target. The expression of EGFR differed greatly among the cell lines (Figure 1A), but it was higher than in NOK in all cell lines except UT-SCC-12A. Survivin is normally not expressed in NOK and was extensively up-regulated in the cell lines, more specifically in eight out of nine (Figure 1B). COX2 was upregulated in five out of nine cell lines. Since the aim of the study was to find a combination of markers correlating to IR, the ARD values of these three proteins were combined, which resulted in a significant correlation to IR ( $R=0.0825$ ,  $p=0.006$ ). However, when COX2 was omitted, the correlation was even stronger ( $R=0.878$ ,  $p=0.002$ ; Figure 1C).

The expression values of the Bcl-2 family proteins (Bcl-2, Bcl-X<sub>L</sub>, Bax, Bad, Bak, and PUMA) are summarized in table III of paper I. Bcl-2 was only up-regulated in UTSCC-34, whereas Bcl-X<sub>L</sub>, Bad, Bax, and PUMA

where overexpressed in several cell lines. The latter three markers all had a negative R-value, consistent with their pro-apoptotic function. Therefore, when the ARD values of multiple markers were simultaneously assessed, anti-apoptotic markers and growth markers were added and the pro-apoptotic markers were subtracted from the total value. Only Bak could positively influence the correlation to IR. When subtracted from EGFR and Survivin, Bak slightly increased the correlation to IR (Figure 2A).

Smad 4, Hsp70 and Cyclin D1 were overexpressed in seven, eight and nine of the cell lines, respectively. Smad 4 and Hsp70 but not Cyclin D1 increased the correlation values when added to EGFR, survivin and Bak (Figure 2B).

The p53 protein was overexpressed in three of the cell lines and its regulator Mdm2 was overexpressed in two (table IV). They did not increase the correlation to IR together with the other markers. Since p53 is of great importance in tumorigenesis it was analyzed on both protein and gene level. The p53 mutations assessed were; splice site, missense and loss of transcript mutations. All cell lines displayed some kind of p53 defect (table II).

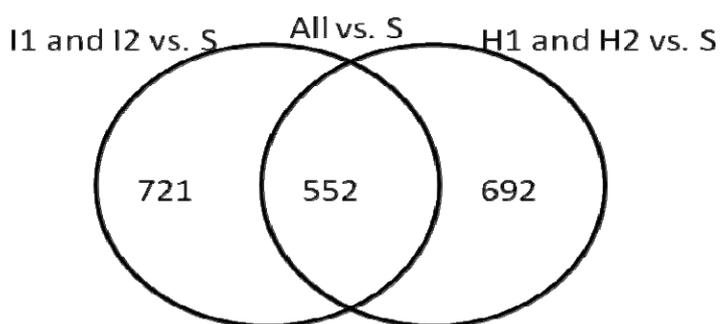
Changes in protein expression and mutations of genes are important events in cancer development. Therefore we created a system where the influence of deregulated protein expressions and p53 mutations could be simultaneously assessed. This was named number of negative points (NNP). We divided the expression of proteins compared to NOK into four groups; No, small, intermediate or large changes (0-3 points respectively), as compared to the expression in NOK. Each p53 mutation received one point. Using a multivariate computer calculation, all possible combinations of factors were analyzed. The combination of factors with the strongest correlation to IR was EGFR, survivin and splice site and missense p53 mutations ( $R=0.990$ ,  $p<0.001$ ; table V).

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

## PAPER II

The use of systematic genomic technologies for construction of models of complex biological systems has increased dramatically during recent years. The aim of such an approach is to bring biological context to the vast amount of data generated in high-throughput analyses like microarrays. In this study we used affymetrix microarrays to generate unsupervised gene expression data, and two bioinformatic tools to relate to the functional importance of the deregulated genes. The aim was to identify the functional key players of intrinsic radioresistance with the purpose of finding predictive markers of radioresponse. The model consisted of five HNSCC cell lines, one sensitive, two intermediately resistant and two highly resistant to radiation.

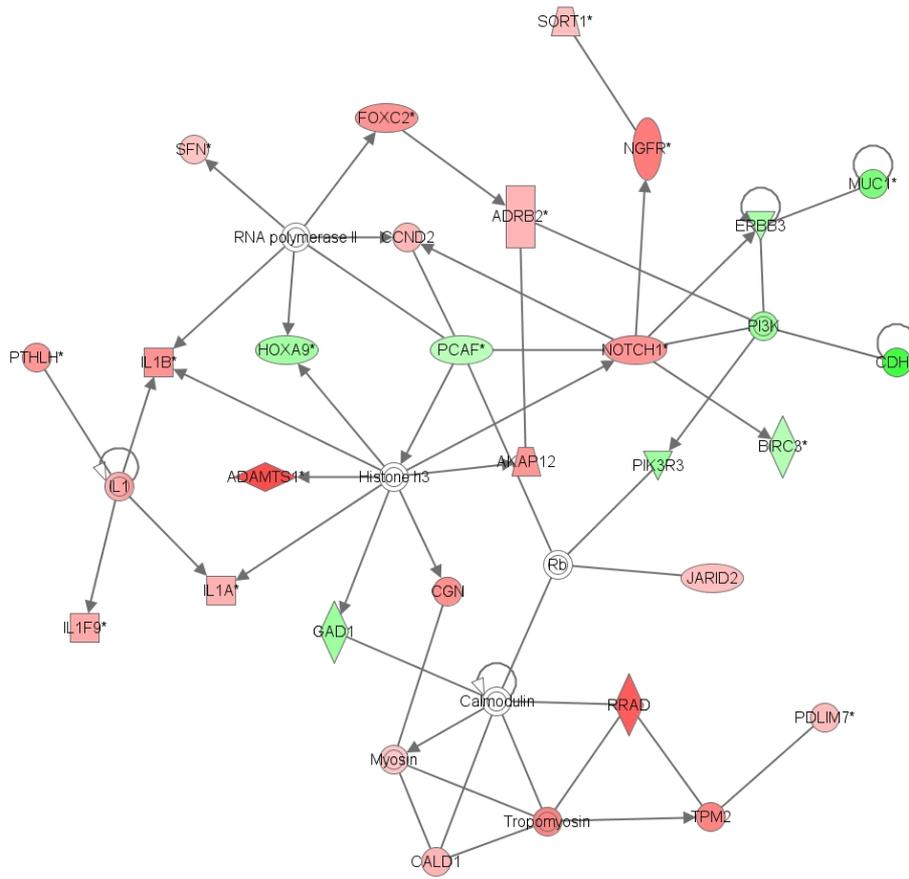
The microarray analysis generated approximately 1300 transcripts that were significantly differently expressed for both intermediately resistant cell lines compared to the sensitive or for both highly resistant cell lines compared to the sensitive. A Venn diagram was generated, in order to elucidate which transcripts that were specific for the two intermediate cell lines, specific for the two highly resistant cell lines or commonly deregulated in all four cell lines as compared to the sensitive. This generated three new gene lists as indicated in Figure 2.



**Figure 2.** Venn diagram of deregulated transcripts in intermediately resistant cell lines (I1 and I2) and highly resistant cell lines (H1 and H2) compared to the sensitive cell line (S) generated three gene lists that were subject to further analysis.

The gene lists generated from the Venn diagram were analyzed for enrichment of gene ontology categories. These results are summarized in table II of paper II. In the highly resistant cell lines, the categories containing the largest number of transcripts are development, negative regulation of biological process, regulation of signal transduction, protein binding, and extracellular region. In the intermediately resistant cell lines, development and immune system process contain many transcripts. Common for all cell lines compared to the sensitive is categories such as cell proliferation, death, development, response to external stimulus, oxidoreductase activity, receptor activity, structural molecule activity, and extracellular region. Studies of GO enrichments and cancer progression in HNSCC have declared deregulation of cell proliferation, development, signal transduction, structural molecule activity, protein binding, and extracellular components (38, 52, 54, 55). GO-analysis of irradiation resistant and sensitive cell lines have proposed cell proliferation and signal transduction as significantly enriched, albeit this was performed in cervical and lung carcinomas (56, 57). Our GO-results are new to HNSCC and intrinsic radiosensitivity.

Transcripts that enriched the GO categories were further analyzed using IPA, which generates molecular networks based on interactions proposed in the IPA knowledge base. This database consists of approximately two million peer-reviewed publications, and is manually curated by PhD-scientists. Apart from the visual molecular networks, IPA also generates a score based on the number of genes in a network along with the top functions of every network. The network with the highest score is shown in Figure 3.



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**Figure 3.** The molecular network (with the highest score) of deregulated transcripts generated by ingenuity pathway analysis (IPA). Key regulators interact with at least three deregulated transcripts as defined by Staab *et al.* Journal of proteome research 2007;6(9):3705-17.

The molecular networks generated were analyzed for key regulator genes, as previously described (52). Since these key regulators have central positions in the molecular networks, i.e. interact with several deregulated transcripts, they are most likely of a higher functional importance than peripherally situated genes. The key regulator genes, top functions and scores of the molecular networks are summarized in tables III-V in paper II. The analysis detected 28 unique key regulators, of which NOTCH1 (notch 1), SERPINE1 (pai-1), and THBS1 (thrombospondin 1) were selected for protein level verification based on the differential expression in the intermediate or highly resistant cell lines compared to the sensitive. The expression levels implied by the transcriptional profile were confirmed by the western blot analysis (paper II figure 1B). All three proteins were present to a

higher extent in the intermediate and highly resistant cell lines compared to the sensitive cell line.

The transcriptional profile of 28 key regulator genes has interesting implications. Notably, we can confirm the role in both HNSCC and radioresponse that seven of these markers have been suggested, that is CTNNB1, ITGB1, NFKB1, NOTCH1, PLAU, SERPINE1, AND TP53 (58-64). The accompanying key regulators are, although some previously mentioned in HNSCC, novel potential biomarkers to IR in head and neck cancer.

NOTCH1, THBS1 and SERPINE1 are promising biomarkers of intrinsic radioresistance, as put forth by this study. In addition, the altered genes, gene ontologies and networks presented herein should likewise be considered as potential biomarkers, although requiring further evaluation.

### **PAPER III**

This study aimed to elucidate markers of cisplatin resistance in three HNSCC cell lines. By microarray analysis we were able to assess all genes that were commonly deregulated in two cisplatin resistant cell lines compared to the sensitive cell line, which generated a list of 781 differently expressed genes. We wanted to examine the functional categories that these genes involve, and analyze key players of the molecular networks of deregulated genes. Using the GOTM tool, this gene list generated 11 enriched categories that are summarized in table II of paper III. Under biological process, development, cell adhesion, cell differentiation, cell migration, and response to virus was detected. The gene ontologies connected to molecular functions were calcium ion binding and interferon binding. Lastly, four categories, extracellular region, extracellular matrix, plasma membrane part, and vacuole, were enriched under cellular component.

IPA analysis generated seven molecular networks in which 20 key regulators were detected (table III paper III). A transcriptional profile was generated from which five potential biomarkers were selected for further analysis, based on their differential expression in the resistant

cell lines compared to the sensitive. These were; APOE, CTNNB1, MMP7, MMP13, and THBS1. The relative expression differences of APOE, CTNNB1, and THBS1 detected in the microarray analysis was confirmed on protein level by western blot analysis. The five markers were all subject to qPCR analysis to evaluate their predictive value in a larger tumor material. Analysis of 25 cell lines did not give significant differences between cell lines expressing high or low mRNA levels of APOE, CTNNB1, and THBS1 ( $p=0.67$ ,  $0.30$  and  $0.27$ , respectively). There was a significant difference in ICS between cell lines expressing high or low mRNA levels of MMP7 ( $p=0.0013$ , Figure 2A). Moreover, MMP13 displayed a strong tendency which was not statistically significant ( $p=0.058$ , Figure 2B). Similar to paper II, we also suggest that the deregulated genes, gene ontologies and networks are potential biomarkers for treatment resistance.

## GENERAL DISCUSSION

Numerous prognostic and predictive markers involved in apoptosis, growth control, DNA-repair and other pathways have been proposed in HNSCC (13, 21). However, none of them has made their way into a widespread clinical use (53), which is still based on histologic appearance, tumor grading, lymph node status, and presence of distant metastasis (12). Since cancer progression involves multiple deregulated functional groups, it is probable that a panel of several markers can reach a higher predictive value. Even though the papers of this thesis use different approaches and methods, this is a central concept upon which they congregate. Using a knowledge based panel of markers chosen from the literature in paper I, and an unsupervised analysis with innovative bioinformatic processing in papers II and III, the aim of establishing predictive markers of treatment resistance is shared.

In paper I, a panel of two expressed proteins (EGFR and survivin) and one mutated gene (p53), showed a high correlation to IR. EGFR and survivin was not detected as predictive markers in paper II. This is explained by the experimental setup, which combined the deregulated transcripts of cell lines in an attempt to encircle the phenotype of intrinsic radiosensitivity. In paper II, the transcripts must be

significantly deregulated in either both the highly resistant cell lines (UTSCC-24 and UTSCC-77) or in both the intermediate resistant cell lines (UTSCC-12 and UTSCC-33) to be included in the analysis. The EGFR expression in Figure 1A and survivin expression in Figure 1B of paper I indicate the differential expression values. However, survivin is implied as an important regulator of pathways such as cell death and cellular proliferation (65), processes that are deregulated in the radioresistant cell lines. A survivin network of cell death markers generated in IPA reveals the involvement of three of the key regulators detected in paper II, namely NFkappaB (NFKB1), p53 (TP53), and beta-catenin (CTNNB1) (19), indicating a functional link between the different markers. TP53 was identified as a key regulator detected in both cisplatin resistant (table III paper III) and radioresistant cell lines (tables III-V paper II) and was one of the markers that in paper I was found to correlate IR. Upon DNA-damage, which is a consequence of both radiotherapy and cisplatin, p53 induces cell cycle arrest and apoptosis, and thereby contribute to the positive effects of these treatments.

The collection of novel targeted therapies proposed in HNSCC is intriguing. However, the majority of cancers resist single molecule directed therapies (19). Many refined and combined treatment regimes, such as altered fractionation and chemoradiotherapy, are also being evaluated. However, if the survival of HNSCC patients is to be considerably higher than today, predictive markers of treatment response must be proposed, validated and implemented into clinical practice.

## CONCLUSIONS

From the results of this thesis, the following conclusions are drawn:

- The NNP method successfully combines protein expression and presence of gene mutations to predict intrinsic radiosensitivity.
- A panel of markers: EGFR expression, survivin expression and p53 mutations, can predict intrinsic radiosensitivity in HNSCC cell lines.
- Deregulated gene ontologies, molecular networks and key regulators were generated for radioresistant and cisplatin resistant cell lines, and are proposed as predictive markers for IR and ICS.
- Transcriptional profiles of possible predictive value for IR and ICS were generated.
- NOTCH1 (notch1), THBS1 (thrombospondin1) and SERPINE1 (pai-1) are suggested markers of IR.
- MMP7 and MMP13 are potential biomarkers of ICS.

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