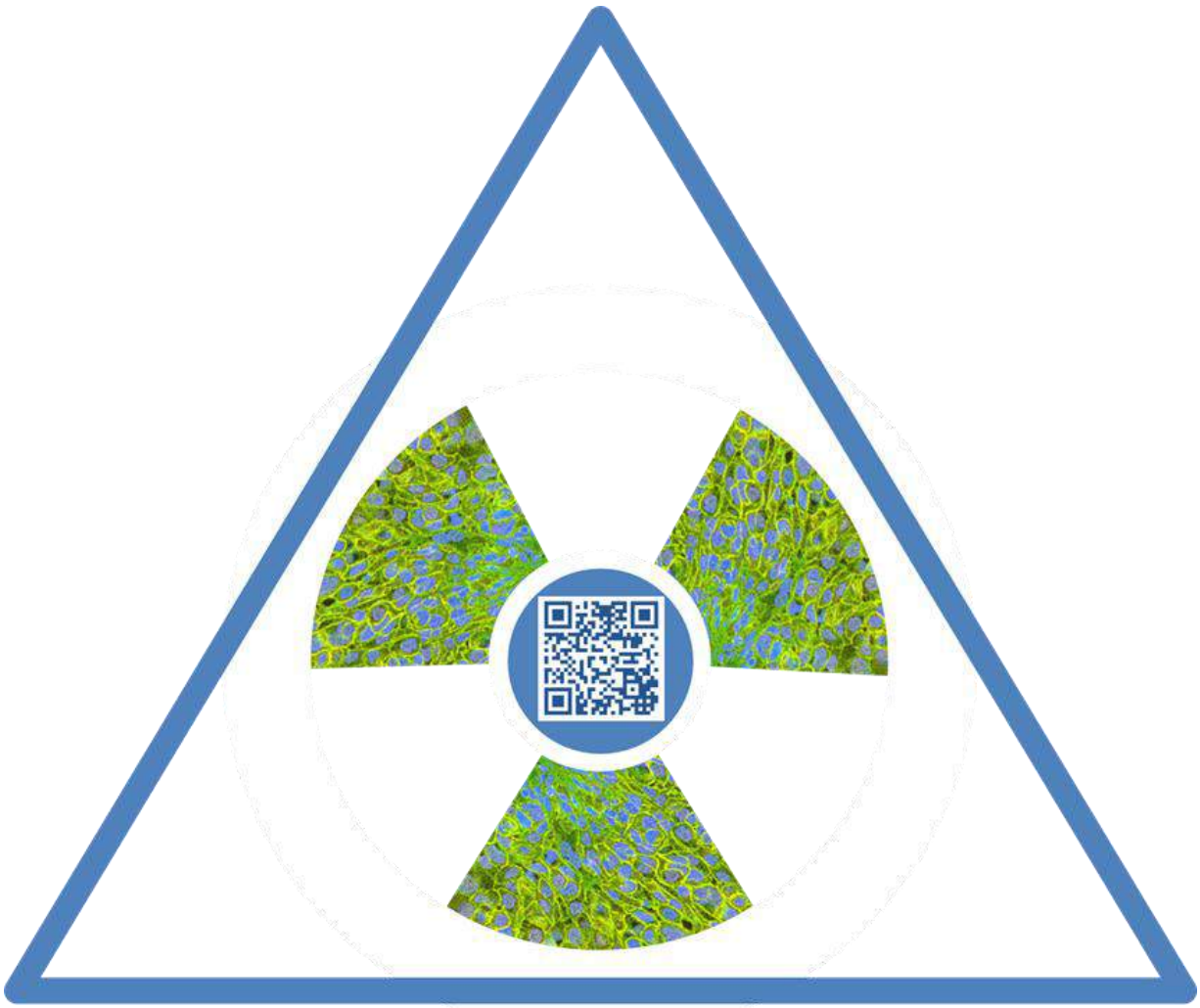
PREDICTING RADIORESISTANCE IN HEAD AND NECK CANCER

Monique C. de Jong



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Predicting radioresistance in head and neck cancer

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geboren te Leiden

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General introduction

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1.1 Cancer

The human body is made up out of trillions of cells (cell size illustrated in a movie). All of these cells have their own tasks to keep the body functioning correctly. This means that some cells are being renewed every couple of days, and others stay where they are for years. Despite the fact that cells from different organ systems can have very different tasks, they all have the same 3 billion deoxyribonucleic acid (DNA) base pairs. Although the DNA of each cell contains the same information, they can use different control mechanisms to prevent and repair damage. Damage to the DNA occurs thousands of times per cell per day due to endogenous and exogenous DNA-damaging factors or during cell division (1). Even though the cellular DNA repair system is very accurate (DNA repair explained in a movie), throughout a person's lifetime most cells will acquire changes somewhere in their DNA. Through these changes it can happen that one of these cells manages to acquire the properties to escape all control mechanisms to become a cancer cell: a cell that does not stop dividing and can grow into other tissues. To become a tumor, cells need to acquire characteristics that enable them to keep proliferating and invading, without being stopped by signaling in or outside the cell as summarized in the hallmarks of cancer by Hanahan and Weinberg (2, 3). The fact that they have updated their review to add more (emerging) hallmarks between 2000 and 2011 (figure 1.1), only shows that we are still in the process of increasing our understanding of how a cancer cell becomes (and stays) a cancer cell.

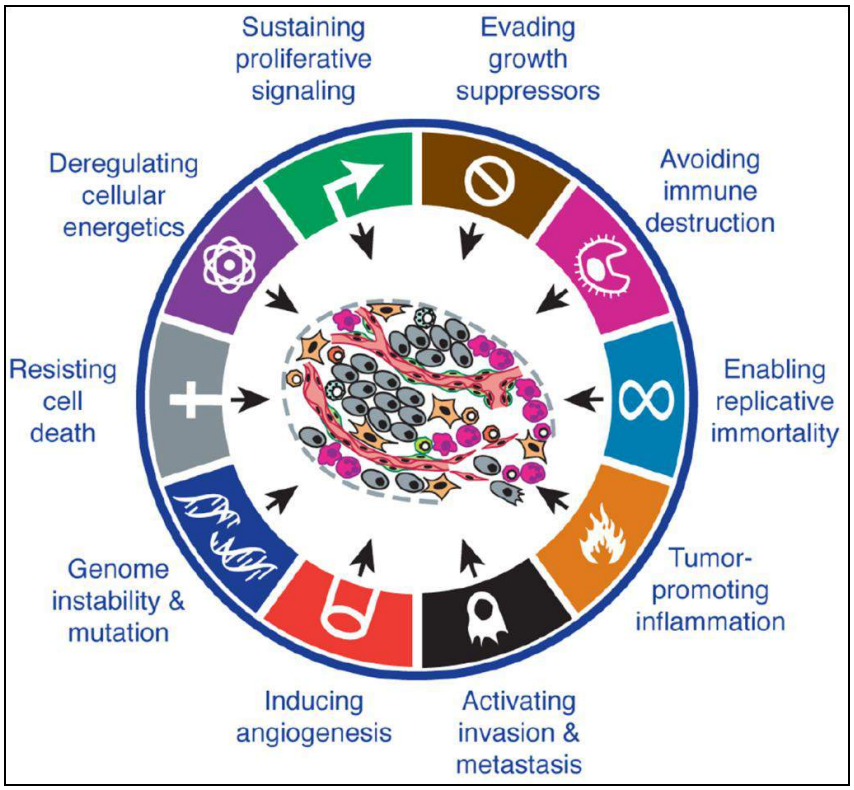

Figure 1.1: (Emerging) hallmarks of cancer, adapted from Hanahan and Weinberg 2011 (2), with permission, © 2011 Elsevier Inc. Published by Elsevier Inc.

1.2 Head and neck cancer

1.2.1 Definition

The term head and neck cancer is used to summarize a group of tumors derived from cells in one of the following subsites of the upper aerodigestive tract: the oral cavity (mouth), oropharynx, nasal cavity, nasopharynx, hypopharynx, salivary glands and the larynx (figure 1.2). Except for salivary gland carcinomas, all tumors are squamous cell carcinomas. In the remainder of this thesis, the nasal cavity, nasopharynx and salivary glands will not be included when head and neck cancer is mentioned.

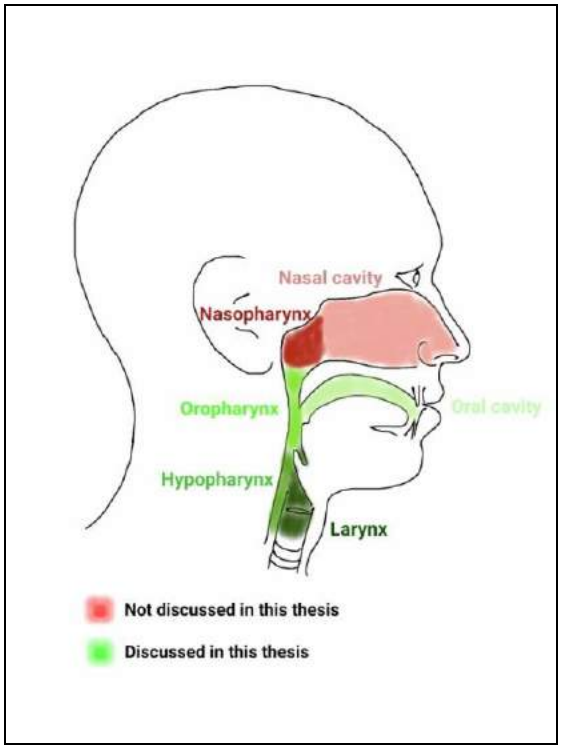

Figure 1.2: Head and neck cancer regions.

1.2.2 Epidemiology

Head and neck cancer is the 7th most common cancer worldwide (figure 1.3). Yearly over 600,000 people are diagnosed with head and neck cancer and over 350,000 people die from it (4). The incidence is generally higher in developing countries (4). From the different subsites of head and neck cancer, cancer of the lip and oral cavity is the most common (44%), followed by cancer of the hypo-/oropharynx and larynx (figure 1.3). The highest incidence of head and neck cancer is in the age group between 55 and 70 years old (5). Head and neck cancers occur predominantly in men, with only 20-30% of all new patients in the US in 2017 expected to be female (calculated from table 1 in ref.(6)).

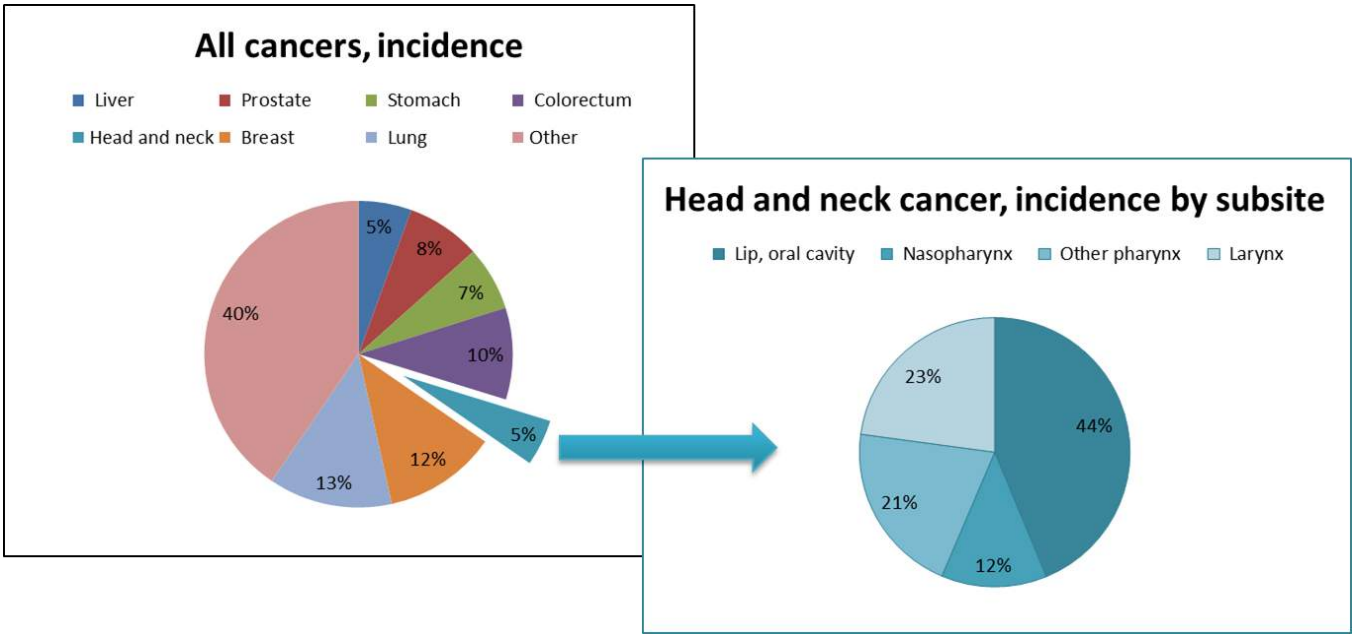

Figure 1.3: Incidence of all 14,067,894 cancer cases worldwide in 2012. Data plotted from GLOBOCAN 2012 data (website) (7).

1.2.3 Etiology

An estimated 75% of all head and neck cancers can be attributed to tobacco smoking and excessive alcohol consumption (8). Heavy smokers or drinkers have a higher risk to develop head and neck cancer, with respective odds ratios of 5 and 2 (9). For people that abuse both alcohol and tobacco, the odds ratio is almost 40, showing that the effect of both substances together is more than additive (8). Another important causative agent is infection with the human papilloma virus (HPV), especially subtype HPV16 (10). The virus produces oncoproteins E6 and E7, leading to tumor initiation. For all tumors the HPV prevalence rate is 26%, this is lower for cancer of the larynx and higher in oropharynx tumors (10). Although HPV infection is very common and in most cases does not lead to the development of cancer, the risk to develop an oropharyngeal tumor increases substantially with HPV16 infection. When comparing patients with oropharyngeal tumors to a group of healthy controls, the HPV16 infection rate is 30-35% versus 0.5-1% in control groups (11, 12). This difference in HPV infection rates can be measured already ten years before the diagnosis of the oropharyngeal tumor (12).

1.2.4 Methods to study a head and neck tumor

There are many ways to study all characteristics of a head and neck tumor: from a simple look in the mouth to an array investigating all biological processes in a tumor (summarized in figure 1.4).

Clinical examination
Clinical examination is used to get an initial impression of the extent of the disease. For head and neck cancer this comprises mainly endoscopic examination of the tumor (youtube) and palpation of the tumor and regional lymph node stations in the neck.

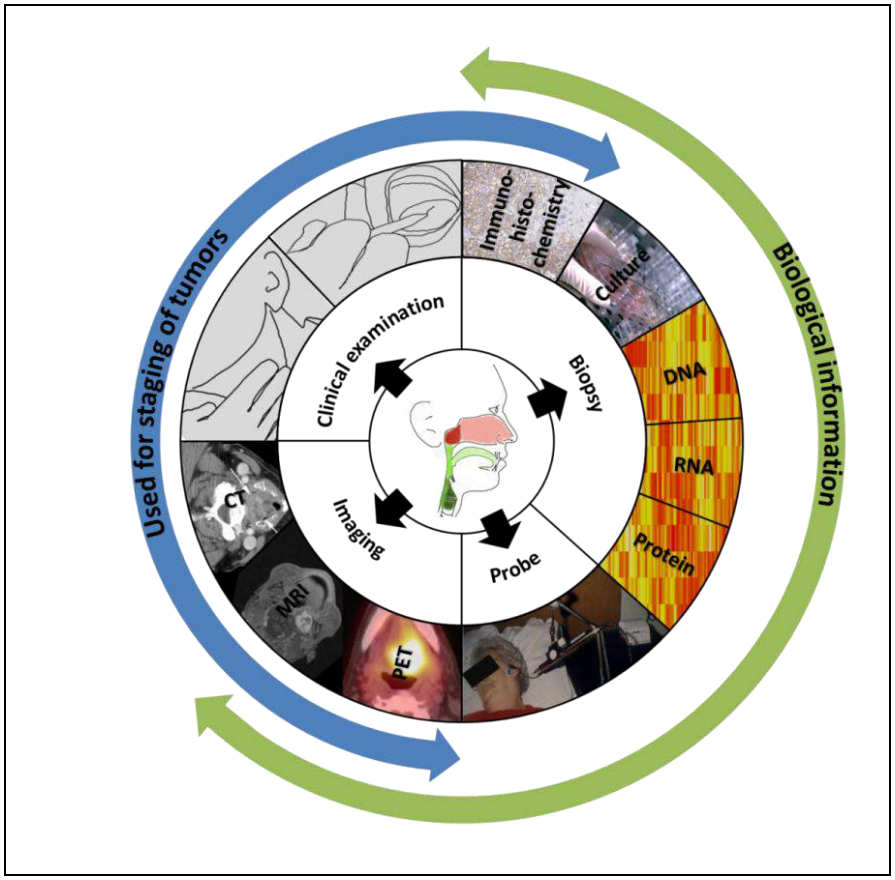

Figure 1.4: Methods to study a head and neck tumor.

Imaging (13, 14)

Ultrasound can be used in combination with a fine needle aspiration biopsy to assess tumor presence in cervical lymph nodes or assess depth of infiltration in oral cancer. *Computed tomography (CT) scanning* is often used to get an overview of the location of the tumor and possible cervical lymph nodes. It is routinely used to make segmentations to determine the extent of the radiation fields or measure tumor volumes. *Magnetic resonance imaging (MRI)* scanning is often used to get a better soft-tissue contrast and can be very helpful to visualize and delineate head and neck tumors. Additionally, different scanning protocols can be used to image different structures more clearly or study different tissue characteristics like diffusion or perfusion.

Another method to produce functional images is *positron emission tomography (PET):* different molecules labeled with a positron-emitter, like fluorine-18 can be used. After injection into the body, the molecule of choice will distribute throughout the body. The best known PET tracer is fludeoxyglucose (FDG), glucose labeled with fluorine-18, used to study glucose uptake in different tissues, which can be used to detect (metabolically active) tumor cells. A clear advantage of PET scanning is the accrual of real-time biological information and the possibility to use a variety of molecules as PET tracer, depending on the process one means to study (15). Examples of tracers other than FDG, that are of particular interest in radiation oncology are thymidine labeled with carbon-11 to measure proliferation, FMISO

([^{18}F]Fluoromisonidazole) and Cu-ATSM for hypoxia imaging and ^{99m}Tc-Labeled annexin to study apoptosis (16).

Radiomics
Apart from anatomical information, recently, different features acquired with these different imaging modalities have been shown to be useful as predictors of outcome (radiomics) (17, 18, 19, 20, 21). The obvious advantage of radiomics is that multiple features can be extracted from standard CT, PET or MRI scans that are already part of the diagnostic or treatment process. The challenge is to correctly place relevant radiomics features in a biological context (21).

Probing
To obtain real-time information about a tumor, a probe can be used to make measurements inside a tumor. The best known are the Eppendorf pO2 measurements with an oxygen sensitive needle probe inserted into the tumor (22). Although this is an invasive technique, it does give access to real time measurements with the possibility to repeat measurements during treatment.

Tumor biopsy
When (part of) a tumor is taken out, the tissue can be studied in a variety of ways. The presence of the HPV virus can be determined on the biopsy material, tumor cells can be grown outside a patient, slices of tissue can be stained and viewed under a microscope and cells or pieces of tissue can be used to study the proteins, RNA or DNA of a tumor or even a single tumor cell (23).

Pathology/Immunohistochemistry
Slices of tumor can be fixed onto glass to study them under a microscope. Different staining protocols, using (fluorescent) dye labeled antibodies, can be used to visualize various markers inside or around tumor cells. This can also be done in a tissue microarray (TMA) format, meaning multiple small slices of tumors from different patients can be stained on the same slide.

Grow cells outside the patient
Cells can be grown in mice (xenografts) or in short- or long term 2D or 3D cultures. This allows researchers to multiply the tumor and to further study the mechanistics of the cells or test the effectiveness of potential therapies. A lecture on 'the good and bad ways' to do this by Adrian Begg can be viewed here: Good and bad ways to assess treatment response .

'Omics'
How (cancer) cells behave is determined by the genetic information stored on approximately 3 billion DNA bases, called the genome (24). A strand of DNA consists of a double stranded sequence of four bases: cytosine, guanine, adenine and thymine. Parts of the DNA can be stimulated to make copies to ribonucleic acid (RNA), a process called transcription. RNA consists of single strands of the bases guanine, uracil, adenine and cytosine, complementary to the transcribed part of DNA. Only a small part (about 1%) of the total DNA contains sequences with exomes (genes), that can be transcribed to messenger RNA (25). This form of RNA is translated to proteins, that will execute the desired actions in a cell (From DNA to

protein in a movie). In total humans have around 20,000 genes (26) and even more proteins because of post-translational modifications (27). The suffix '-omics' stands for a method acquiring a lot of data about all genes in one experiment (Introduction to 'omics' by NASA), which is possible on DNA, RNA and protein levels (figure 1.5). The simultaneous study of the whole genome (DNA) from one sample is termed genomics (28, 29, 30) (NASA explains genomics). Using new techniques to study the whole genome, all cancer-related mutations, translocations, amplifications and deletions can be examined and correlated with outcome or treatment response. Functional genomics studies what kind of processes are active in a tumor (cell) (31). Proteomics, the study of all proteins that are present in a tumor (cell), would presumably best represent what is occurring in that cell at a given time point. However, the study of all proteins in one sample is challenging and less sensitive than other approaches (27, 31). These other approaches include epigenetics, the study of manipulation of DNA to express certain genes, and transcriptomics, the study of messenger RNA that is translated to proteins (32, 33, 34) (NASA explains transcriptomics). For many genes there is a good correlation between messenger RNA and protein levels and an even better correlation between groups of messenger RNAs and certain biological processes. Still, messenger RNA does not always translate into protein. One of the reasons for this is the presence of microRNAs: small pieces of single stranded RNA (around 22 nucleotides) that can singlehandedly silence hundreds of genes (35). Almost 1,000 microRNAs have been identified so far, regulating at least 60% of all genes (36, 37). MicroRNAs regulate gene expression by binding to their (partly) complementary sequence on messenger RNA molecules, finally resulting in reduced protein production (38). MicroRNAs can reduce messenger RNA levels or directly reduce protein levels by translation inhibition, multiple modes of silencing seem to exist, that can be active at the same time (39, 40).

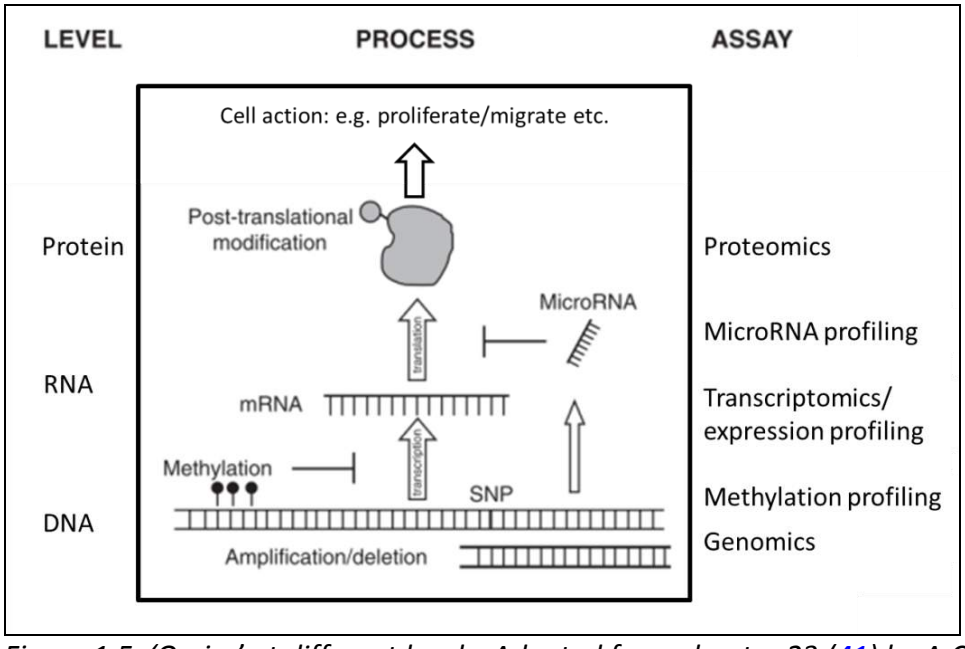

Figure 1.5. 'Omics' at different levels. Adapted from chapter 23 (41) by A.C. Begg.

Frequent genetic defects in head and neck cancer

A median of 5 mutations per megabase was found in a group of head and neck cancer patients (42). Although no two tumors have the exact same genetic defects, there are some

faults that are common amongst different head and neck tumors (43). It has to be stated that tumors caused by HPV do not possess all the same genetic alterations (44 ,45, 46, 47) and might be considered a different entity from tumors that are mainly caused by smoking and drinking (48, 49). Some of the most commonly described genetic alterations in head and neck cancers are in the p53, CDKN2A (p16), CCND1 (cyclin D1), epidermal growth factor receptor (EGFR), PIK3CA and NOTCH pathways (47, 50) as can be seen in figure 1.6.
A loss or mutation of **TP53** on chromosomal location 17p13 can lead to decreased apoptosis and increased proliferation. This is observed in about 50-80% of head and neck cancers (47, 51, 52). The cyclin-dependent kinase inhibitor 2A (**CDKN2A**) gene produces p16, which inhibits CDK4 and CDK6 to prevent phosphorylation of the Rb protein, leading to inhibition of cell cycle progression from G1 to S-phase. In 80% of head and neck cancers the p16 protein is absent, mostly by deletion of the gene location of p16 on chromosome 9p21, which leads to increased proliferation. A study by van der Riet et al, showed that p16 was deleted in 70% of head and neck tumors (53). **Cyclin D1,** on the other hand, activates Rb, thus enabling the transition from G1 to S phase. An activating polymorphism of this gene was described in 25% of tumors (54), whereas the chromosomal region of this gene (11q13) was amplified in 20-50% (43, 55). Another often described oncogene is the epidermal growth factor receptor (**EGFR)** located on chromosome 7p11, which is a regulator of tumor cell growth, invasion, angiogenesis, and apoptosis. This receptor shows overexpression in 40% of tumors (56). Reasons for overactivation of EGFR signaling can be the expression of mutant EGFRvIII (57) or an amplification of the EGFR gene (58). Not just EGFR, but other genes in the **PI3K-AKT** or **RAS-MAPK** can be deregulated, giving the same effect. More recently, inactivation of the **NOTCH** pathway has been found in head and neck tumors. Inactivation of this pathway could lead to loss of regulation of several processes like self-renewal capacity, cell-cycle exit, and survival (59, 60).

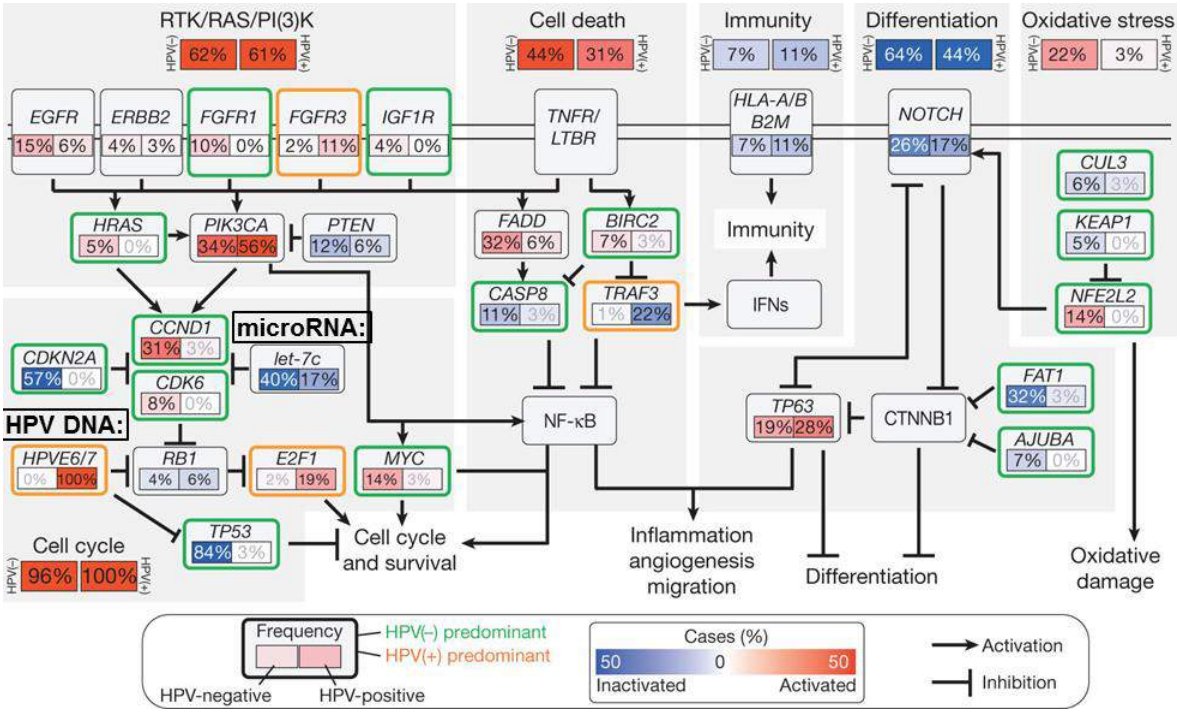

Figure 1.6: Common alterations in 279 HNSCC samples and their role in the different 'hallmarks of cancer'. Picture adapted from Lawrence et al. (47) with permission according to Nature Publishing Group guidelines.

1.2.5 Staging

Based on the spread and extent of the tumor, determined by clinical examination and imaging, patients are classified into different stage groups. Staging can then be used to select the correct treatment or make an estimation of the prognosis. The staging of tumors of the larynx, oropharynx, lip and oral cavity is done using the TNM AJCC Cancer Staging Manual, for this thesis the seventh edition was used, summarized in table 1.1. This edition has recently been updated to the eighth edition (61).

TNM staging of the larynx, oropharynx, lip and oral cavity. Summarized from AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer New York, Inc.		
Primary tumor (T)		
Tx	Cannot be assessed	
T0	No evidence of primary tumor	
Tis	Carcinoma in situ	
Primary tumor (T) Larynx		
T1	**Supraglottis:**	1 supraglottis subsite, mobile vocal cords
	Glottis:	T1a: one vocal cord, T1b: both vocal cords.
	Subglottis:	Limited to the subglottis
T2	**Supraglottis:**	> 1 subsite of supraglottis or other region (e.g., glottis, tongue, vallecula) no larynx fixation
	Glottis:	Supra- or subglottic extension, and/or impaired vocal cord mobility
	Subglottis:	Extends to vocal cord
T3	Limited to larynx with vocal cord fixation	
	Supraglottis:	and/or invades: postcricoid area, preepiglottic space, paraglottic space, thyroid cartilage
	Glottis:	and/or invasion of paraglottic space/ thyroid cartilage
T4a	Invades through the thyroid cartilage (or cricoid in subglottis) and/or tissues beyond the larynx	
T4b	Tumor invades prevertebral space, encases carotid artery, or invades mediastinal structures	
Primary tumor (T) Lip/ Oral cavity/ Oropharynx		
T1	< 2 cm	
T2	> 2 cm, < 4 cm	
T3	> 4 cm. **Oropharynx:** > 4 cm or in epiglottis	
T4a	**Lip:** invasion through cortical bone, inferior alveolar nerve, floor of mouth, or skin of face.	
	Oral cavity: invades adjacent structures only (e.g. through cortical bone, into deep muscle of tongue, maxillary sinus, skin of face).	
	Oropharynx: invasion in the larynx, extrinsic muscle of tongue, medial pterygoid, hard palate, or mandible.	
T4b	Invasion in pterygoid plates, skull base and/or encases internal carotid artery.	
	Lip/oral cavity: invasion in masticator space.	
	Oropharynx: invasion in lateral pterygoid muscle, pterygoid plates, lateral nasopharynx, or skull base or encases carotid artery.	
Regional lymph nodes (N)		
Nx	Cannot be assessed	
N0	No regional lymph node metastasis	
N1	1 ipsilateral node, < 3 cm	
N2a	1 ipsilateral node > 3 cm, < 6 cm	
N2b	Multiple ipsilateral lymph nodes	
N2c	Contralateral nodes	
N3	Lymph node > 6 cm	
Distant metastasis (M)		
Mx	Cannot be assessed	
M0	No distant metastasis	
M1	Distant metastasis	

Table 1.1: Staging of head and neck tumors. Summarized from AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer New York, Inc.

1.2.6 Management

According to the specific (sub-)site and the stage, a patient with a head and neck tumor will be treated with one or a combination of these modalities: surgery, radiotherapy, photodynamic therapy, chemotherapy and targeted therapy (62, 63). Usually treatment consists of radiotherapy, chemoradiotherapy or surgery with or without adjuvant (chemo-)radiotherapy. While surgical resection of the tumor can be effective in terms of tumor control, it is challenging to spare some important functions like speech and swallowing in advanced stages. Therefore a lot of research has been conducted into organ-sparing strategies using either single-modality radiotherapy or radiotherapy in combination with chemo- or targeted therapy. Data from small randomized trials suggest that outcome rates between surgery and (chemo-) radiotherapy are similar, with a possible exception for oral cavity and advanced laryngeal cancers (64, 65, 66). Results of phase II/III studies for the subgroup of small (T1-2) tumors are still awaited (67). Meanwhile, comparable survival rates between surgery and radiotherapy groups were reported in a large literature review of oropharyngeal cancers (68). Generally, less toxicity is reported in these studies when surgery is avoided. Currently, approximately two third of all patients is (partly) treated with radiotherapy: 58% of all patients with pharynx/oral cavity tumors and 74% of all patients with larynx tumors (69).

1.2.7 Prognosis

The average overall survival for head and neck cancer is around 50% (4), but this can vary greatly between groups of patients with different characteristics. One way to divide patients into different prognosis groups is to use the TNM stage groups (table 1.2). The 5 year survival can range from 83% for stage I patients to 30% for stage IV patients (table 1.2) (5, 70).

Stage groups	T	N	M	Prognosis (5 year relative survival rates)
Stage I	1	0	0	50-83%
Stage II	2	0	0	46-62%
Stage III	3	0	0	23-55%
	1-3	1	0	
Stage IV	4	any	any	
	any	1-2	any	22-43%
	any	any	1	

Table 1.2: Stage groups. Summarized from AJCC Cancer Staging Manual, Seventh Edition (2010) published by Springer New York, Inc. Five year relative survival rates from SEER data.

The first sign of failure after therapy is usually a locoregional recurrence (80-90%, calculated from: (71, 72, 73)). The rate of second primary tumors is significantly higher in head and neck cancer patients than for other tumors. Eventually, 36% of these patients will get a second primary tumor (mostly lung cancer). Roughly 10% of all patients will get a second primary head and neck cancer (74), because HPV, smoking and drinking affect the entire

area (field cancerization). Both recurrences and second primaries are challenging to (re-) treat, toxicity is high and the five year overall survival only 20% (75).

1.2.8 Quality of life

During treatment the acute dose-limiting toxicity is mainly severe (grade 3-5) mucositis, occurring in approximately 30% of radiotherapy patients and in 40-70% of accelerated radiotherapy or chemoradiotherapy patients (76, 77, 78, 79). In a systematic review that summarizes quality of life data from 37 studies among head and neck cancer survivors, toxicity at one year after treatment was reported (80). Persisting issues reported at that time were mostly fatigue, xerostomia (dry mouth) and sticky saliva. Other observed symptoms at 1 year were problems with appearance, speech, swallowing, taste/smell and sexuality. Primary hypothyroidism has also been described as a late complication after treatment of tumors of the head and neck (81).

1.3 Radiotherapy for head and neck cancer and reasons for its failure

While in daily clinical practice the TNM staging system is used to predict prognosis and base treatment decision on, the failure of radiotherapy treatment can be attributed to factors on different levels. The treatment, patient characteristics, tumor characteristics and cell properties can all contribute to the eventual cure or failure (summarized in figure 1.7).

1.3.1 Treatment characteristics

Radiotherapy

Radiotherapy using photons causes damage through the generation of free radicals or through direct damage in the cell. Radiation damage causes various DNA defects, of which the most lethal is the DNA double strand break. An illustration: a typical fraction dose of 2 Gray (Joule/kilogram) induces > 2,000 DNA base damages, ~2,000 DNA single strand breaks, and 40-80 DNA double strand breaks per cell (82). Every 2 Gy-fraction will kill around 30-50% of the tumor cells. Fractionated radiotherapy uses the principle that normal tissues have a better ability to repair (DNA) damage than tumor cells, and will therefore (partly) recover in between fractions, while tumor cells will not. The relative advantage of a treatment course integrating both the tumor and the normal-tissue effects can be expressed in a therapeutic ratio (41). Alterations in the radiotherapy fractionation or the addition of chemotherapy or targeted therapies aim to specifically target tumor cells and thereby improve the therapeutic ratio. An example of a typical curative head and neck irradiation schedule is 35 fractions of 2 Gy (total dose 70 Gy) over 7 weeks.

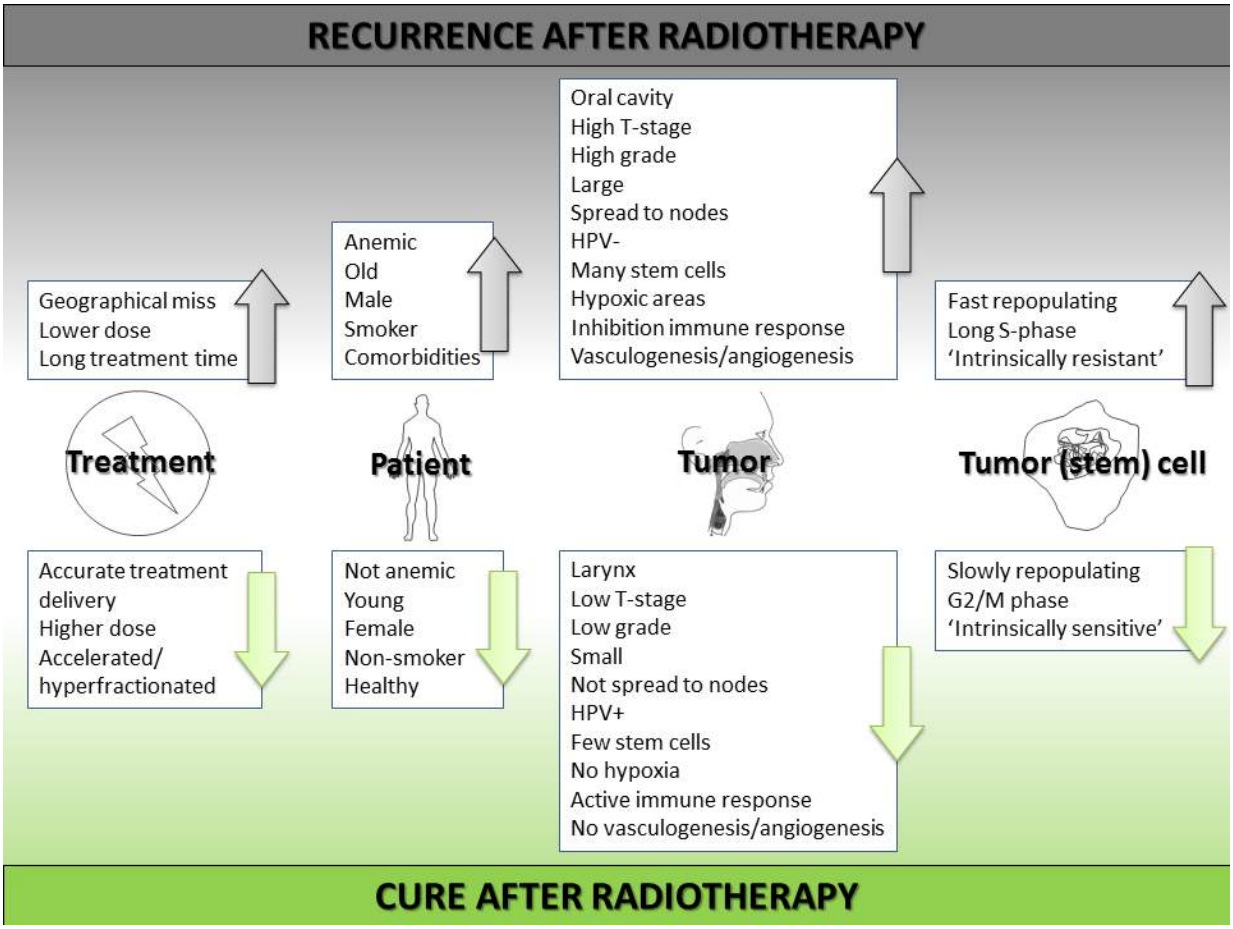

Figure 1.7: Overview of causes of radiotherapy failure (upwards arrows) or success (downwards arrows) in head and neck cancer.

To make different irradiation schedules comparable in terms of the biological effect, treatment schedules can be recalculated to the equivalent dose in 2-Gy fractions (EQD_2) using the Linear Quadratic (LQ) model (Lecture LQ model by Adrian Begg). Assuming the overall treatment time is unchanged the formula is: $EQD_2 = D * (d + \alpha/\beta)/(2 + \alpha/\beta)$, were D is the total dose, d the dose per fraction and the α/β-ratio represents the fractionation sensitivity of the tissue of interest (for head and neck cancer 10 is commonly used). An EQD_2 converter can be found here: EQD_2 converter. When the overall treatment time is changed (usually to accelerate the treatment) the EQD_2 is calculated as follows: $EQD_{2,t.new} = EQD_{2,t.old} - (t.new - t.old) * D_{prolif}$, where *t.new* is the new overall treatment time in days, *t.old* the original treatment time in days and D_{prolif} is the dose recovered per day due to proliferation; for head and neck cancer this is 0.7 Gy/day (83).

Treatment and treatment delivery

Different treatment-related factors can contribute to treatment failure. The total dose (EQD_2) has been shown to predict survival (84, 85). A higher total EQD_2 gives a better tumor control, but is sometimes compromised because of an interruption of the treatment. Other reasons for a lower EQD_2 are concessions due to dose limiting normal tissue toxicity (important but not discussed in this thesis) or missing part of the tumor extent on pretreatment imaging leading to a lower dose or complete geographical miss of the tumor, meaning part of the tumor will not receive the total dose needed for tumor kill. Time plays

an important role in head and neck cancer radiotherapy. Both the overall treatment time and the time to treatment initiation have been shown to be important predictors of outcome. A delayed start of treatment is prognostic ally unfavorable (86, 87). Data from the US National Cancer Database show that patients with a waiting time under 52 days have a median overall survival of 72 months, versus 47 months for patients with a waiting time over 67 days (86). Radiotherapy can further be improved by using accelerated (reduction of total treatment time) or hyperfractionated (more fractions in the same treatment time) treatment schedules (72, 79, 88). A lecture by Jack F. Fowler on hyperfractionated and accelerated radiotherapy can be viewed here: altered fractionation (Jack F. Fowler, 1989). The benefit of these regimens is an absolute overall survival benefit of 3.4% at 5 years (8.2% for hyperfractionation). Other strategies to improve radiotherapy outcome are the addition of chemotherapy (89) or targeted therapy (90). Concomitant chemotherapy gives an absolute survival advantage of 6.5% at 5 years in a large meta-analysis (91). On the addition of targeted therapies to radiotherapy, there are no meta-analyses yet. So far, a combination of radiotherapy with the hypoxic sensitizer (nimorazole) (92) or the EGFR-inhibitor cetuximab have shown promise (90). More targeted therapies are currently under investigation, as well as proton therapy (93).

1.3.2 Patient characteristics

Patient-related factors

Many patient characteristics have been described to influence cure and survival rates. Factors that have been linked to decreased survival are a higher age, male sex, pre-treatment anemia, a poor general health/comorbidity and (persistent) smoking. **Older patients** do worse that younger patients; this has been shown in several studies with an average hazard ratio of 1.5 per decade (84, 85, 94, 95, 96). **Male sex** was reported to have a hazard ratio of 2.3 compared to female sex in a series of 994 laryngeal cancer patients that were treated with radiotherapy (84). Patients with a **low hemoglobin** concentration before start of radiotherapy have a worse overall and disease free survival rate, with a hazard ratio of around 1.4 for patients with anemia (84, 85, 97). The worse survival of anemic patients cannot be overcome by a transfusion prior to the start of treatment (98). Patients with a **worse general health** score either defined as performance status (85, 99), ASA comorbidity score (100) or ACE-27 score (101) do worse than healthy patients without comorbidities (102). In a large study conducted to identify behavioral factors that influence survival of head and neck cancer patients, **being a former or current smoker** gave a decreased overall survival (with respective hazard ratios compared to never smokers of 2.0 and 2.4) (95). Molina et al. reported a slightly lower hazard ratio of 1.3 for tobacco use (94). In a recent study by Gillison et al. the risk of death increased by 1% per pack-year that was smoked (103).

Tumor-related factors

Tumor properties that have been described to influence cure rates negatively are a higher T and/or N-stage, a large tumor volume, the site from which the primary tumor originates and biological characteristics. A higher **T stage** is correlated with a worse overall survival, with estimated hazard ratios of 1.5, 2 and 3 for T2, T3 and T4 tumors compared to T1 tumors (73, 84, 85, 96, 97, 101). Another way to describe the primary tumor is the measurement of the primary tumor volume on pre-treatment imaging (CT/MRI/PET). The larger the **tumor**

volume, the worse overall survival rates, this was demonstrated in a few studies that measured tumor volume, the overall survival rate was reported to decrease around 10% for every 10 cm^3 volume increase (100, 104, 105, 106, 107). In all of these studies, the addition of tumor volume to a multivariate model eliminated T-stage as a significant predictor of overall survival. The extent of lymph node involvement, the **N stage** is often correlated with survival, in a study by Schroeff et al. 5 year survival was 61.3% for N0 and 10% for N3. Others have reported similar findings (84, 85, 96, 97, 99, 101, 104), with hazard ratios compared to N0 for respectively N1,N2 and N3 patients, being around 1.5, 2 and 3. Different studies show the importance of **tumor subsite** for the prediction of outcome (84, 94, 95, 97, 99). A representative example is the study by Schroeff et al., which showed that in a large population cohort, patients with a glottic larynx tumor had a much better 5 year survival (68%) than other sites like oral cavity (42%), oropharynx (37%) or patients with hypopharynx (28%) tumors (101).

1.3.3 Tumor biology

The survival of patients can be influenced by general prognostic biological factors like tumor grade or HPV status, but also by predictive factors that are (partly) specific for the response to radiotherapy (108). The **grade** of the tumor is a measure for its aggressiveness that correlates with prognosis. In a study by Molina et al. a moderate to poor differentiation grade has a hazard ratio of 1.2 over good differentiation (94, 101). Fairly recently the **HPV infection status** has been discovered to be a major factor for the prediction of outcome of head and neck cancer (109). Patients with HPV-positive tumors have a reduction in the risk of dying from their cancer when compared with HPV-negative tumors. In a meta-analysis of 37 studies by Ragin et al. a 28% reduced risk of death was observed (hazard ratio 0.72) (110). In three studies of patients treated with (chemo-)radiotherapy a consistent 60% reduction in the risk of death was observed (hazard ratio 0.4) (111, 112, 113). The superior cure rates of patients with HPV positive tumors might be caused by an increased sensitivity to irradiation due to impaired DNA repair (114, 115). Because of their superior survival, patients with HPV positive tumors have even been suggested to be candidates for treatment deintensification (49, 116, 117).
Classical radiobiological processes influencing tumor response to irradiation are oxygenation, proliferation and intrinsic radiosensitivity (118), also described as the 4 or 5 'Rs': **R**epair, **R**eoxygenation, **R**epopulation, **R**edistribution of cells in the cell cycle and intrinsic **R**adiosensitivity (119, 120). More recently other processes have been added to these factors: the presence of stem cells, microenvironmental factors like blood supply and immune cells and possibly also the energy metabolism of the tumor cells (121, 122).

Repair
The term repair, or recovery, is often interpreted as 'DNA repair', but was originally (before the discovery of DNA repair) used to describe the observation that tissues can recover after radiotherapy. This recovery has different aspects: repair of DNA damage (discussed under 'intrinsic radiosensitivity') and tissue factors (discussed under 'microenvironmental factors').

(Re-) oxygenation
Hypoxic cells treated with radiotherapy have a survival advantage. This was shown by numerous *in vitro* studies (among others: (123, 124, 125, 126)). The fact that hypoxia is a

negative prognostic factor, has also been shown *in vivo*, using different techniques to evaluate the level of hypoxia in a tumor (127). Hypoxia can be measured directly by invasive methods or indirectly by imaging techniques or by studying protein or messenger RNA expression of genes known to be involved in hypoxia (127). Of note is that hypoxia is often subdivided into chronic (diffusion limited) and acute (perfusion limited) hypoxia, which of these two has the most implications for therapy outcome is still under debate (128). Many methods to study hypoxia in a tumor exist, consisting of invasive methods, different imaging techniques and various analyses of biopsy material (129). Direct, pre-treatment Eppendorf pO2 measurements with an oxygen sensitive needle probe inserted into the tumor, demonstrated that a high percentage of hypoxic areas within the tumor was associated with poor survival (22, 130, 131, 132). Studies of PET imaging of hypoxia with different tracers indicated that, again, hypoxia correlates with worse control rates after radiotherapy (133, 134, 135, 136). Hypoxia PET scans can also be of use in the monitoring of hypoxia during treatment: a decrease of hypoxic tumors from 70-100% before treatment to 6-36% during treatment was observed (133, 137, 138). Another imaging strategy to study hypoxia is MRI, using specific scanning protocols, like dynamic contrast enhanced (DCE) MRI (129, 139, 140). The most extensively immunohistochemically studied hypoxia markers are the exogenous pimonidazole and the endogenous markers HIF1-alpha and carbonic anhydrase IX (CAIX). Pimonidazole (an exogenous compound preferentially bound by hypoxic cells) staining correlated with local control after radiotherapy: 2-year local control rates increased from 48% to 87% when pimonidazole staining decreased (141). Overexpression of HIF1-alpha, a proposed marker for acute hypoxia, correlated significantly with worse local control (142, 143, 144), as well as expression (pattern) of CAIX, a HIF-1alpha target and pH regulator (142, 145, 146, 147). With the notion that one marker might not reflect the complex cellular response to hypoxia, there have also been reports of panels of markers (gene expression sets) studied simultaneously that correlate hypoxia status with outcome (148, 149, 150, 151).
Finally, the fact that *in vivo* modification of oxygen status during radiotherapy can improve local control, especially in hypoxic tumors, proves that hypoxia is an important factor in radioresistance (92, 133, 138, 151, 152, 153).

Repopulation/proliferation (Link to lecture by Adrian Begg on proliferation)
Using fractionated radiotherapy, not just normal tissues, but also tumors have the opportunity to compensate for their loss, meaning fast proliferating tumors will (partly) renew themselves in between fractions. Two factors are of importance for this phenomenon: the ability to proliferate quickly and the number of cells that have clonogenic capacity (154). The potential tumor doubling time, measured on pre-treatment biopsy material, was a significant predictor in single center studies, but failed to show a significant correlation with outcome in a multicenter validation study of 476 patients (155). However, in head and neck cancer, a negative effect of prolongation of overall treatment time has been shown. From about 5 weeks after the start of fractionated radiotherapy an accelerated repopulation has been observed, meaning that with a longer overall treatment time, more dose is needed for the same tumor control rates (156, 157, 158). This observation has been used to design new fractionation schedules. When the same dose (70 Gy) was administered in 6 weeks instead of 7, a significantly higher tumor control rate (around 10% higher) was observed (79, 159). However, not all patients appear to benefit from accelerated radiotherapy, additional subgroup analyses have shown that the benefit is for patients with

a well differentiated, slowly proliferating tumors with high EGFR expression (160, 161, 162, 163). An explanation for this counterintuitive finding could be that these tumors resemble normal mucosa and therefore still share the ability for accelerated repopulation (164). Another approach to measure proliferation of a tumor, could be to detect the glucose uptake (165). Tumor uptake of 2-[(18)F] fluoro-2-deoxy-D-glucose (FDG) measured by positron emission tomography (PET) has been shown to be a prognostic factor in a series of 120 head and neck cancer patients. A higher glucose uptake (measured by a higher standardized uptake value) was correlated with worse disease free survival (166).

Redistribution

Over 50 years ago it was observed that cells in different phases of the cell cycle showed different survival rates after irradiation (167, 168). It was shown that cells are generally more sensitive to irradiation during mitosis/G2 phase and more resistant during the (late) S phase. Fractionating radiotherapy would increase the probability of irradiating cells in a more sensitive phase, because of the redistribution in phases in between two fractions (119, 169). In series of in head and neck cancer patients treated with differently fractionated radiotherapy schedules, it was observed that tumors with a longer duration of S phase (measured *in vitro*) had worse local control rates: around 30-40% in tumors with a longer S phase, compared to 50-60% for tumors with a shorter S phase duration (155, 170).

Intrinsic (cellular) radiosensitivity

Within a tumor, different cell populations exist, with different sensitivity to irradiation (171). Tumor cell radiosensitivity, defined as the sensitivity of cells to ionizing radiation *in vitro*, is a significant prognostic factor for radiotherapy outcome (118). The sensitivity of cells *in vitro* can be tested by measuring clonogenic survival at specific doses of irradiation. The percentage of surviving colony-forming cells at a certain dose level can then be determined. Survival of cells at 2 Gy was shown to correlate with tumor control rates in studies that compared *in vitro* cellular radiosensitivity to therapy response (172, 173, 174, 175). Hypothetical causes for cellular radiosensitivity can be divided into three categories: *1.* Cells get less damaged upon irradiation, *2.* Cells repair DNA damage better/faster after irradiation, *3.* Cells with the same amount of damage have better pro-survival mechanisms. Although there is not much evidence for the first hypothesis, it has been suggested that cells with more radical oxygen species scavengers, like glutathione, have higher survival rates (176, 177). Another possible factor contributing to the evasion of damage from radiotherapy is chromatin density. Areas of more condensed chromatin have been shown to be less prone to double strand breaks (178, 179). The second hypothesis, better DNA damage repair, is probably the most important and most investigated explanation for intrinsic sensitivity. Cells that are defective in DNA repair are more sensitive to irradiation. This can be learned from patients with DNA repair disorders (180, 181). Luckily, in most cancer patients, impaired DNA-repair is specific to tumors, which leads to improvement of the therapeutic ratio of fractionated radiotherapy. Numerous *in vitro* experiments have shown a radiosensitization after the inhibition of one of the DNA repair pathways (128, 182, 183). Some drugs targeting the DNA damage response are currently tested in clinical phase I/II studies (184). A lecture on the exploitation of DNA repair by Adrian Begg can be viewed here: Exploiting DNA repair to improve radiotherapy. Finally, the ability to evade death after getting damaged by irradiation could contribute to cells being more resistant. Firstly, by the correct activation of cell cycle checkpoints upon obtaining DNA damage, a cell can take the time to repair damage

and thereby evade mitotic catastrophe. There is evidence that cell cycle checkpoint inhibition can lead to higher tumor control rates (185). In case the damage is too extensive, there are many ways for a cell to die (186, 187). Although the most researched method, apoptosis through TP53 signaling, is not consistently linked to radiosensitivity, other modes of dying could be correlated with radiosensitivity (188, 189). There is some evidence in head and neck cancer that TP53 does not inhibit apoptosis, but causes treatment failure by evasion of senescence (190).

Other processes (not starting with an 'R')

Since the 4 or 5 classic 'Rs' have been defined decades ago, there are some new insights as to why tumors can be radioresistant. Firstly, the discovery that not all cells in a tumor are important for the survival of that tumor gave rise to the characterization of the cancer stem cell model (Lecture Professor Weinberg on cancer stem cells): only some cells in a tumor are able to regrow a new tumor and are therefore the only cells that need to be killed in order not to get a tumor recurrence after radiotherapy (191, 192). This means that all other factors (all classic 'Rs') are only important for those cancer stem cells (121). There is a growing body of evidence suggesting that not only the percentage of cancer stem cells in a tumor is important, but that cancer stem cells are intrinsically more radioresistant than non-cancer stem cells (193). Secondly, there is a growing recognition that the microenvironment in which a tumor cell grows is important for its response to irradiation. The microenvironment can influence radiotherapy response in several ways. Cancer cells can be influenced by their neighboring cells, leading to the bystander effect (indirect damage of initially undamaged cells because they are next to irradiated cells) (194). Another important component of the microenvironment is the vasculature. Tumor often have a 'messy' vasculature leading to various levels of hypoxia. Additionally, endothelial cells dying as a response to radiotherapy can prevent the regrowth of tumor cells that were being supplied by that vessel (195). The inhibition of vasculogenesis has been shown to prevent tumor recurrence in glioblastoma xenografts (196). Other important cells in the microenvironment are the immune cells. Infrequently an abscopal radiotherapy effect is observed: stimulation of the immune system by irradiation of one tumor location can stimulate immune cells to eradicate tumor cells at an unirradiated site in the same patient (197). Given recent breakthroughs in cancer immunotherapy, there is a growing interest in the stimulation of this abscopal effect by combining radiotherapy with immunotherapy (198, 199, 200, 201, 202). Lastly, the altered energy metabolism of tumor cells can have an effect on radiosensitivity; a different redox state can lead to more ROS scavenging or have an effect on immune invasion or angiogenesis (122, 203, 204).

Prediction of response to radiosensitizers

Biological properties are not only useful to predict response to radiotherapy, but also response to radiosensitizers. It has been shown that pre-treatment tumor hypoxia status can predict benefit from hypoxia-sensitizers added to radiotherapy (92, 133, 138, 151, 152, 153). Response to concurrent cisplatin could be predicted by measuring cisplatin-DNA adduct levels or loss of nuclear p53 signal (205, 206) and a worse response to EGFR inhibitor has been attributed to activation of ERK signaling, KRAS mutations or the absence of the KRAS-variant (207, 208, 209, 210).

1.4 Thesis outline/aim/scope

Clearly there is a need for the improvement of survival rates in head and neck cancer, ideally with a reduction of severe toxicity. The most likely way to accomplish this is to better select patients for a treatment that fits their specific tumor characteristics. Currently only the clinical characteristics are used and treatment is based on site and TNM stage, which merely account for 25% of the variation in survival (211, 212, 213, 214).
The research described in this thesis aims to discover more about the individual biological tumor properties in head and neck cancer, using messenger- and microRNA data to predict which tumors will be more radioresistant and why. Eventually this could lead to a better understanding of the reasons for radiotherapy failure and an up-front adaptation of therapy (depicted in figure 1.8) to give each individual patient the best chance of survival (215, 216, 217).

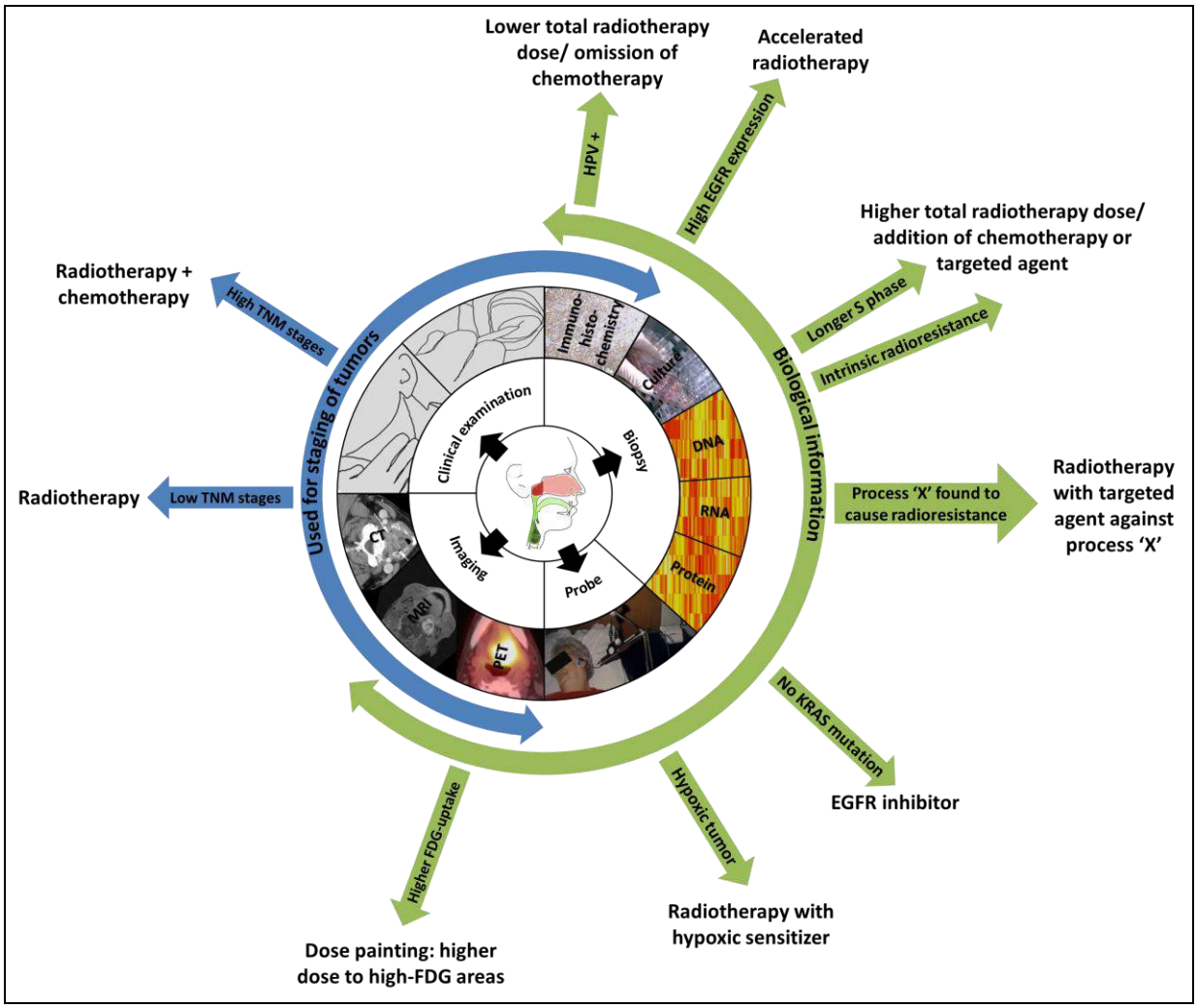

Figure 1.8. An example of the current use of clinical factors (blue) and how addition of biological knowledge could individualize and improve radiotherapy (green).

In chapter 2, we show that gene expression can improve the prediction model and adds valuable information to known clinical factors for local control after chemoradiotherapy in 75 advanced head and neck cancer patients. Chapter 3 describes the analysis of a more homogeneous series of 52 T1-2 larynx cancer patients, treated with single modality radiotherapy. Pre-treatment high expression of the putative stem cell marker CD44 correlates with local recurrence rate in this training series and in an independent validation cohort of 76 patients. Chapter 4 describes the discovery of an intrinsic radioresistance gene set on mRNA and micro RNA expression data from 32 head and neck cancer cell lines. We found that low expression of miR-203, giving more epithelial-to-mesenchymal transition, not only corresponds with intrinsic radiosensitivity, but also predicts outcome after radiotherapy in larynx cancer patients. Chapter 5 describes the comparison of published hypoxia gene sets that seem very dissimilar. However, these almost entirely different sets of genes classify 224 head and neck cancer patients nearly identically.

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(Hyperlinks to references in text)

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CHAPTER 2

HPV and high-risk gene expression profiles predict response to chemoradiotherapy in head and neck cancer, independent of clinical factors

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Abstract

PURPOSE:
The purpose of this study was to combine gene expression profiles and clinical factors to provide a better prediction model of local control after chemoradiotherapy for advanced head and neck cancer.

MATERIAL AND METHODS:
Gene expression data were available for a series of 92 advanced stage head and neck cancer patients treated with primary chemoradiotherapy. The effect of the Chung high-risk and Slebos HPV expression profiles on local control was analyzed in a model with age at diagnosis, gender, tumor site, tumor volume, T-stage and N-stage and HPV profile status.

RESULTS:
Among 75 patients included in the study, the only factors significantly predicting local control were tumor site (oral cavity vs. pharynx, hazard ratio 4.2 [95% CI 1.4-12.5]), Chung gene expression status (high vs. low risk profile, hazard ratio 4.4 [95% CI 1.5-13.3]) and HPV profile (negative vs. positive profile, hazard ratio 6.2 [95% CI 1.7-22.5]).

CONCLUSIONS:
Chung high-risk expression profile and a negative HPV expression profile were significantly associated with increased risk of local recurrence after chemoradiotherapy in advanced pharynx and oral cavity tumors, independent of clinical factors.

 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer world wide, with almost 650,000 new cases and 350,000 disease related deaths annually [1]. At presentation, around half of these patients have advanced disease [2]. In this group there is a limited benefit from radiotherapy alone (5 year locoregional control 12.6-37.4%) [3]. Combined with chemotherapy, higher locoregional control rates of up to 65% can be achieved [4, 5, 6, 7, 8, 9]. However, the obvious benefit due to the addition of chemotherapy comes at the cost of higher grade III-IV toxicity. It is therefore essential to predict which patients will not benefit from chemoradiotherapy, which patients will become disease free, and in this last group, which patients would have been disease free with radiotherapy only.

Currently, clinical factors such as stage, site and tumor volume are used to predict response and select treatment [10, 11, 12, 13, 14, 15, 16, 17, 18, 19]. In the largest series analyzed so far, Knegjens et al. found tumor volume to be the most important predictor of outcome after chemoradiotherapy [20]. Like Knegjens, Chen et al. found a poorer outcome for patients with primary tumors above 30 cc [21]. However, the predictive power of clinical factors is still limited.

Apart from clinical factors, infection status with high risk Human Papilloma Virus (HPV) should be taken into account. HPV-associated tumors have a different pathogenesis, with different and less chromosomal aberrations than tumors caused by alcohol and tobacco abuse [22]. HPV-positive tumors arise more often in the oropharynx than in other sites. Patients with these tumors seem to have a better prognosis than HPV-negative patients [23, 24, 25, 26].

In recent years, gene expression profiling has been used to search for gene signatures correlating with outcome. These have the potential to provide insight into mechanisms and can monitor multiple biological processes. To date, such gene signatures as a single factor have shown prognostic potential [27, 28, 29].

Chung et al. [30, 31] found a gene expression profile containing mostly genes involved in epithelial-mesenchymal transition and NFκB pathway activation. This profile was highly prognostic for survival in two series of head and neck cancer patients treated with primary surgery with or without adjuvant therapy. This signature was subsequently validated in an independent dataset by Pramana et al. [32], who tested the signature in a series of HNSCC patients treated with combined radiation and cisplatin, with locoregional control as the endpoint. It therefore appears to be predictive in this setting, but its independence of clinical factors was not evaluated.

In this study, we further investigated whether a HPV profile (published by Slebos et al. [33]) and the Chung profile are able to add predictive power to the current prediction of local recurrence with just clinical factors.

Materials and methods

Patients

Of 92 advanced HNSCC patients with gene expression data available, patients were eligible for analysis in the current series if they had a stage III/IV (M0) tumor and there was a good quality MRI or CT scan on which to measure the primary tumor volume. In the previous analysis by Pramana et al. [32], oral cavity and larynx cancer patients were excluded from the final analysis because they showed very different survivals after treatment and could therefore have confounded the effect of gene expression. For the current analysis, we decided to include oral cavity tumors, since we aimed to study whether the effect of gene expression was independent of clinical factors. Larynx cancer patients were not deemed representative for this study population because according to the Dutch Consensus guidelines they do not usually receive chemoradiotherapy [34].

Treatment

All patients were categorized as anatomically or functionally inoperable and treated with curative intent. Treatment consisted of cisplatin-based concomitant chemoradiotherapy regimens in phase II/III studies at the Netherlands Cancer Institute. The different schedules all included irradiation with 70 Gy in 35 fractions over 6-7 weeks. Chemotherapy was administered either intra-arterial (i.a.) 150 mg/m^2 on treatment days 2, 9, 16 and 23, intra-venous (i.v.) daily low dose (6 mg/m^2) cisplatin or intra-venous on treatment day 1, 22 and 43 (100 mg/m^2). There was no significant difference in outcome between intra-arterial and intra-venous chemoradiotherapy [35].

Chung gene expression profile

The methods for generating expression profiles have been described previously [32]. Briefly, gene expression profiles were measured on pre-treatment biopsies of all patients. Different published gene sets were tested, of which a “high risk” signature published by Chung et al. [31] was the most significant predictor of locoregional recurrence. Unigene identifiers were used to map the 42 Chung genes to the latest annotations of the NKI array. When more than one probe mapped to the same Unigene cluster, the probe with the least missing values and with the highest interquartile range (IQR) was used. This resulted in 32 genes to be used for analysis. For each patient, Pearson correlations were calculated against the Chung score. Patients were grouped into those who had a negative or positive correlation of their gene expression values with the high risk Chung profile, representing a predicted low or high risk, respectively.

HPV profile

Since there was no DNA available to test for infection with HPV, gene expression was used to asses HPV infection status. Slebos et al. published a set of 20 genes that were upregulated when HPV is transcriptionally active [33]. Symbols for these genes were updated from the NCBI Entrez Gene database (www.ncbi.nlm.nih.gov/sites/entrez), and the corresponding probe numbers on the NKI array selected. In this way, 12 of the 20 genes could be mapped

to the NKI array and were used as the HPV signature (table 2.1). When more than one probe mapped to the same gene, the probe with the least missing expression values across the patient series and with the highest interquartile range (IQR) of expression between the patients was used. Since only upregulated genes were used, average expression of these genes was calculated for every patient and the median of the average expression values used to divide patients into two groups, the group with low HPV gene expression (under the median) being considered HPV negative-like and the group with high HPV gene expression being considered HPV positive-like.

HPV gene signature	
Gene symbol	**Description**
C16orf75	C16orf75 protein
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CENPK	Centromere protein K
EHHADH	Peroxisomal bifunctional enzyme
MCM6	DNA replication licensing factor MCM6
MYNN	Myoneurin
NR1D2	Orphan nuclear receptor NR1D2
RFC4	Replication factor C subunit 4
RIBC2	RIB43A-like with coiled-coils protein 2
RPA2	Replication protein A 32 kDa subunit
SYNGR3	Synaptogyrin-3
TAF7L	TATA box binding protein-associated factor

Table 2.1. HPV gene signature: The 12 upregulated genes from the Slebos study [33] that could be mapped to our microarray platform and were used to determine HPV profile status.

Tumor volume

The pretreatment CT or MRI scan was used for primary tumor volume measurement. All visible primary tumor was manually delineated on every CT or MRI slice. Pathological lymph nodes were not included. Tumor volume was calculated after triangulation of the surface of the delineations [20].

Statistics

The primary endpoint for this study was local control. A local recurrence was defined as a pathologically proven recurrence at the site of the primary tumor. Time to local recurrence was calculated from the date of diagnosis until local recurrence, death, loss to follow-up or end of follow-up, whichever occurred first. Events other than local recurrence resulted in censoring of time to local recurrence. The association with local control was evaluated for gender, age at diagnosis, primary tumor site, T and N-stages, primary tumor volume, Slebos HPV expression status and Chung gene expression status by Kaplan-Meier plots and corresponding log-rank tests as well as by hazard ratios (HR) and 95% confidence intervals (CI) based on Cox regression. Age at diagnosis was dichotomized at the median among

patients with a recurrence; tumor volume was dichotomized using a 30 cc cut off. Trend tests were based on the slope of the continuous variable. Variables with a HR>1.5 or <0.5 or a p-value<0.05 for at least one category in univariate analyses were included in a multivariate model. Kaplan-Meier curves were generated in GraphPad PRISM 5.01. All other analyses were performed using SPSS 15.0. Based on the results of the multivariate analysis, patients were grouped according to their total number of independent risk factors for local recurrence.

Comparison with a larger series

The present dataset was limited to patients who had available gene expression data. To assess reproducibility of the results found for clinical factors, we compared our results to the results of a series of 360 patients also treated with radiation plus cisplatin and from which 75% of the present study patients were taken [20].

Results

Patient inclusion

Of 92 patients, 75 were eligible for analysis in the current series. A total of 17 patients were excluded from further analysis for the following reasons: 10 patients had a T1-2 or larynx tumor, 1 patient was a double entry, 1 patient had a volume of nearly 400 cc, more than 4 times higher than the next largest tumor, and was therefore not considered to be representative of the group, and 5 patients had a poor quality CT scan and therefore no volume data could be obtained. Tumor volume was measured on MRI scans for 64 patients and on CT-scans for 11 patients.

Patient characteristics

The characteristics of the patients are shown in table 2.2. The study population was predominantly male (69%) with a mean age at diagnosis of 58 years. Patients had a pharynx tumor (oropharynx and hypopharynx combined) in 85% and a tumor of the oral cavity in 15%. The mean primary tumor volume was 30.9 cc, ranging from 4.3 cc to 96.7 cc. Patients received radiotherapy with i.a. cisplatin (34 patients), high dose i.v. (18 patients) or low dose i.v. (23 patients) cisplatin treatment. For the Chung status, 64% of the patients were predicted to be low risk and 36% high risk. Since the median average expression for the Slebos HPV genes was used to generate two groups, half of the patients had a positive profile. Median follow-up time was 93 weeks. A total of 17 local recurrences occurred during follow-up, with a median time to recurrence of 24 weeks.

Patient characteristics			
Characteristic	**Categories**	**N**	**%**
Gender	male	52	69
	female	23	31
Age at diagnosis (years)	mean	57.5	
	range	29.1 - 77.3	
Tumor site	oropharynx	47	63
	hypopharynx	17	23
	oral cavity	11	15
T-stage	T3	28	37
	T4	47	63
N-stage	N0	17	23
	N1	10	13
	N2	43	57
	N3	5	7
Primary tumor volume (cc)	mean	30.9	
	range	4.3 - 96.7	
Chung risk profile	low risk	48	64
	high risk	27	36
Slebos HPV profile	positive	37	49
	negative	38	51

Table 2.2. Patient characteristics: Baseline characteristics of the 75 patients that were included in this study.

Univariate analysis

Of all factors included in the univariate analysis, significant predictors of local recurrence were Chung status, tumor site and HPV profile (table 2.3). Kaplan Meier curves for local recurrence for these factors are shown in figure 2.1. There was no significant difference between hypo- and oropharynx tumors, and so these were combined into one group of pharyngeal carcinomas. Associations with age at diagnosis, T-stage and tumor volume were suggestive, but did not reach statistical significance ($p<0.05$). Oral cavity tumors, a Chung high risk profile and a negative HPV profile were significantly associated with a higher risk of local recurrence.

Univariate and multivariate analysis – local recurrence							
			Cox proportional hazards model				
Variable	**Categories**	**N (no. of events)**	**Univariate**			**Multivariate**	
			HR (95% CI)	p-value	p-value trend	HR (95% CI)	p-value
Gender	male	52 (11)	1.0				
	female	23 (6)	1.2 (0.4 - 3.3)	0.7	-		
Age at diagnosis (years)	<62	52 (9)	1.0			1.0	
	>62	23 (8)	2.5 (1.0 - 6.6)	0.06	0.08	2.7 (0.8 – 9.0)	0.1
Tumor site	oro- and hypopharynx	64 (10)	1.0			1.0	
	oral cavity	11 (7)	6.3 (2.4 - 16.8)	<0.001	-	4.2 (1.4 – 12.5)	0.009
T-stage	T3	28 (3)	1.0			1.0	
	T4	47 (14)	3.1 (0.9 - 10.7)	0.08	-	1.8 (0.5 – 6.9)	0.4
N-stage	N0-1	27 (6)	1.0				
	N2-3	48 (11)	1.0 (0.4 - 2.8)	0.9	-		
Primary tumor volume (cc)	<30	46 (8)	1.0			1.0	
	>30	29 (9)	1.9 (0.7 - 4.8)	0.2	0.1	1.4 (0.5 - 3.9)	0.6
Chung risk profile	low risk	48 (5)	1.0			1.0	
	high risk	27 (12)	5.2 (1.8 - 14.7)	0.002	0.002	4.4 (1.5 – 13.3)	0.008
Slebos HPV profile	positive	37 (4)	1.0	0.03	0.06	1.0	0.006
	negative	38 (13)	3.6 (1.2 - 11.1)			6.2 (1.7 – 22.5)	

Table 2.3. Univariate and multivariate analysis – local recurrence: Results of the univariate Cox proportional hazards analysis for all factors. The hazard ratio (HR) between the two categories of each factor is given, together with the p-value and, if applicable, a p-value for the trend of the corresponding continuous variable. Results of the multivariate Cox proportional hazards analysis for the five factors with a HR>1.5 or <0.5 or a p-value<0.05 in the univariate model.

Multivariate analysis

Of the six factors entered in a multivariate Cox regression, tumor site, Chung status and HPV status were significantly associated with local control (table 2.3). Patients with oral cavity tumors were 4 times as likely to get a local recurrence compared to patients with a pharynx tumor (HR 4.2, 95% CI 1.4 – 12.5). Risk for local recurrence was increased at a similar magnitude for patients with a Chung high risk signature compared with the low risk group (HR 4.4, 95% CI 1.5 –13.3). Patients with a HPV-negative profile were 6 times more likely to get a local recurrence than patients with a HPV-positive profile (HR 6.2, 95% CI 1.7 – 22.5).

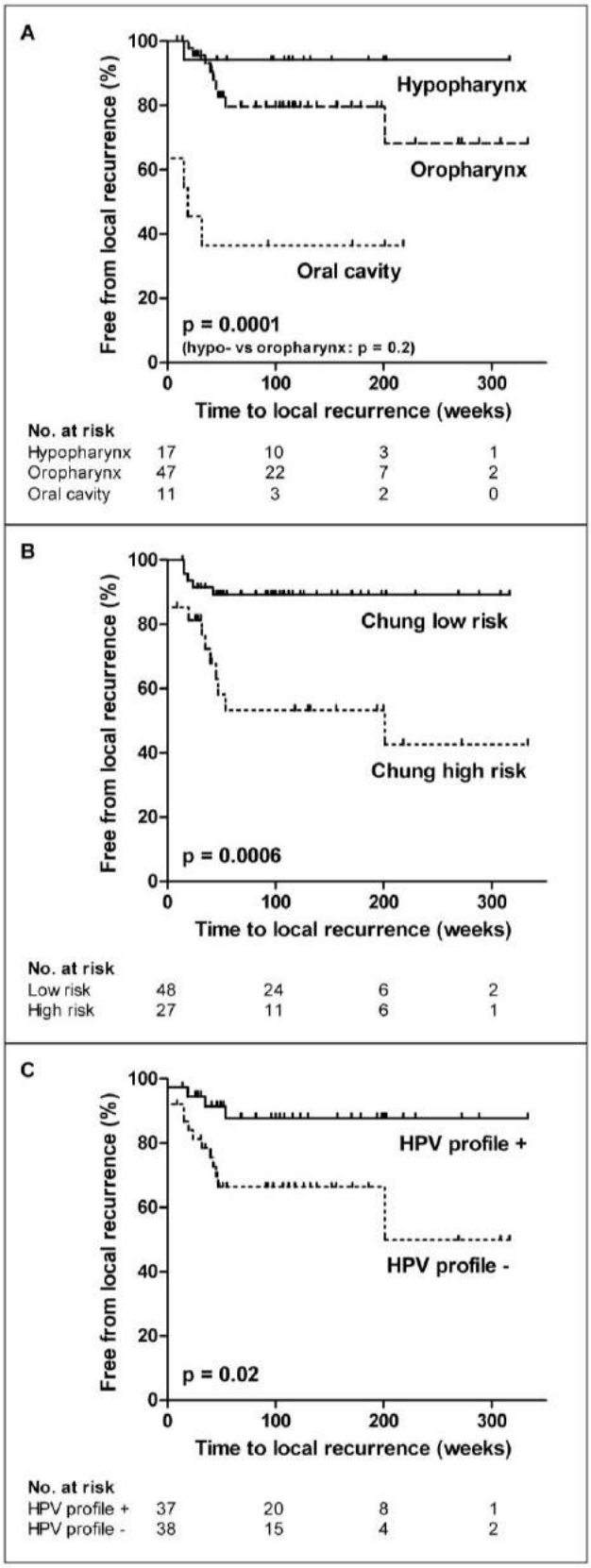

Figure 2.1. Site, Chung and HPV profile: Kaplan-Meier curves for all 75 patients grouped by based on site (A), Chung risk group (B) and HPV profile status (C). The given p-values were calculated with a log-rank test.

Local recurrence by number of risk factors

Figure 2.2 shows a Kaplan-Meier curve for a combined model of site, Chung status and HPV status. The number of unfavorable features (an oral cavity tumor, a Chung high risk profile and a HPV-negative profile) were added up for every patient. For example, a patient with a tumor of the pharynx with a Chung low risk profile and a HPV-positive profile has 0 high risk features. From this figure can be seen that in the group of 22 patients with just favorable factors (0) there were no recurrences during follow up and the 4 patients with three unfavorable factors all had recurrences.

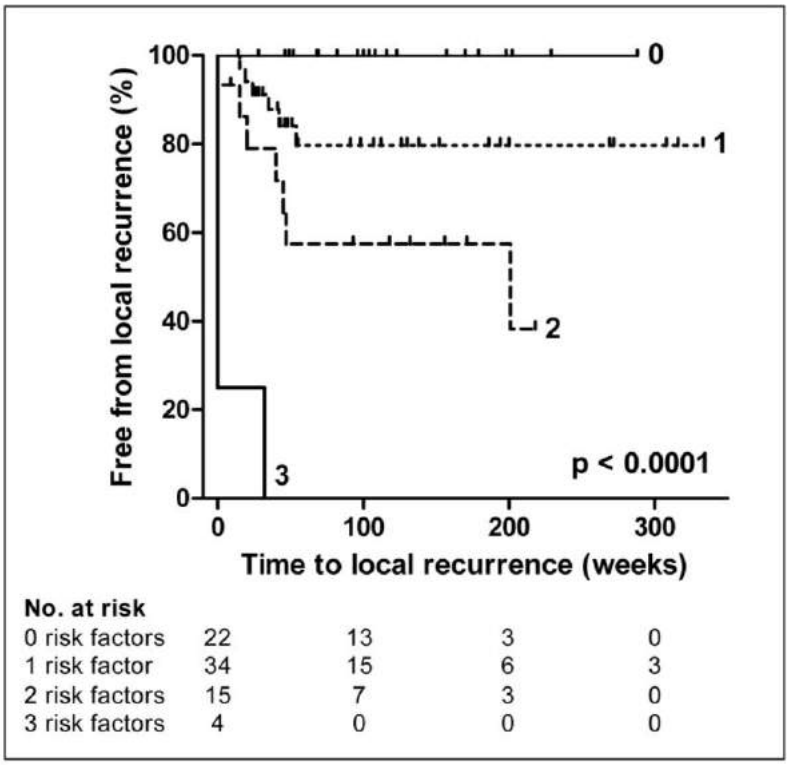

Figure 2.2. Local recurrence by number of risk factors: Kaplan-Meier curve for all 75 patients organized into groups based on the number of high risk features (Chung high risk profile, HPV profile negative and oral cavity). The given p-value was calculated with a log-rank test.

Comparison with a larger series

In line with the results for the series of 360 patients [21], site and, to a lesser degree, T-stage were important predictors of local control. Tumor volume was not significantly associated with local control in the univariate analysis in the current study, whereas the association was highly significant in the earlier published larger series of 360 patients ($p<0.001$) [21] from which the present patient population was taken. However, the magnitude of the association was approximately similar, but was attenuated in the multivariate analysis of the current data. We explored the dependence of the strength of association on sample size by drawing ten random samples of 75 patients from the series of 360 patients (table 2.4). Tumor volume was significantly associated with local control in 5 of the 10 samples, and 3 of the 10 corresponding p-values exceeded the one observed in the current smaller series. The differences observed for tumor volume is therefore likely due to the smaller size of our

current series. In addition, it is possible that the Chung or HPV profiles partly capture the tumor volume signal in the multivariate analysis.

Random series of N=75 from N=360											
Series	1	2	3	4	5	6	7	8	9	10	No of series with p<0.05
p-value for volume:	0.004	0.3	0.02	0.08	0.002	0.008	0.04	0.2	0.07	0.2	5

Table 2.4. Random series of N=75 from N=360: Ten series of 75 patients, all randomly selected from a larger series of 360 patients. Five of the ten randomly generated series had a p-value<0.05 for tumor volume in a cox proportional hazards model.

Discussion

Our aim was to study the independence of a high risk and a HPV gene expression profile for predicting local recurrence, when analyzed in a model with known clinical predictors in advanced HNSCC patients treated with chemoradiotherapy. A gene expression profile designed by Chung et al. [31] was previously validated to predict locoregional recurrence after chemoradiotherapy on a series of 92 advanced HNSCC patients by Pramana et al. [32]. From this series we analyzed 75 patients to test association of clinical factors and gene expression with local control. The main finding of this study was that the two gene expression profiles had an independent effect on local recurrence in a model with clinical factors and were the most important independent factors in a multivariate model, together with tumor site. This implies that they could in the future be a valuable addition to the clinical factors that are currently used for prediction of local recurrence.

In this study, it was not possible to test for HPV presence in DNA and therefore, gene expression was used to identify patients with a HPV-like profile. As shown in studies that used DNA tests for HPV, patients with a HPV positive profile had a better cure rate [24, 25]. Lassen et al. and van den Broek et al. showed that high $p16^{INK4A}$ expression (immunohistochemistry) independently predicted good treatment response and survival in patients with head and neck cancer treated with conventional (chemo-) radiotherapy [23, 36]. In their most recent paper, Lassen et al. showed that p16 positive patients do not seem to react to hypoxic modification during radiotherapy [37]. P16 (CDKN2A) was also one of the genes we analyzed with the Slebos HPV profile. To our knowledge, our study is the first to show that a HPV gene set can predict local recurrence.

We are not aware of any other externally validated gene expression signature predicting local recurrence in head and neck cancer patients treated with (chemo-) radiotherapy. Other authors have searched for profiles able to predict recurrence in head and neck cancer [27,

28, 29]. Ginos et al. studied 41 surgically treated patients, in which they found genes that correlated with recurrent disease. None of those genes correlated with site, grade or stage [28]. Ganly et al. found 2 genes predictive of locoregional recurrence after chemo-radiotherapy in 35 patients, using a 277-gene cDNA array [29]. Dumur et al. found 142 genes predictive of locoregional recurrence in 19 patients treated with radiotherapy with or without chemotherapy [27]. The clinical factors they studied (age, gender, stage and location) were not significant in a univariate analysis and therefore no multivariate analysis was performed.

The Chung and HPV profiles are therefore, to date, the only validated signatures for prediction of local recurrence in HNSCC patients. In addition, the present series is the first to be large enough to test independence of validated signatures from clinical factors in a multivariate model. As can be seen in figure 2.2, a combination of site, Chung expression profile and HPV profile, leads to a subgrouping of patients, where the best group has no local recurrences and the worst group has no cures in it. Although the patient numbers were not very high, these kind of subgroups could be very useful to select patients for therapy. The value and robustness of this combination will need to be confirmed in independent studies.

The present study indicates that gene expression signatures can add valuable additional information to current clinical predictors. In future randomized trials, expression profile measurements can thus be useful in indicating which patients benefit most from the treatment being tested, and thus lead to more rationale and effective application of new therapies.

Conclusion

Gene expression profiles can be useful for predicting local control, independent of clinical factors, after chemoradiotherapy in advanced pharynx and oral cavity tumors. Together with tumor site, the Chung high risk signature and HPV profile status were the most important predictors of local control.

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(Hyperlinks to references in text)

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CHAPTER 3

CD44 expression predicts local recurrence after radiotherapy in larynx cancer

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Read commentary 'CD44: A cancer stem cell-related biomarker with predictive potential for radiotherapy' by Michael Baumann and Mechthild Krause in the same issue of Clinical Cancer Research.

Abstract

PURPOSE:
To find molecular markers from expression profiling data to predict recurrence of laryngeal cancer after radiotherapy.

EXPERIMENTAL DESIGN:
We generated gene expression data on pre-treatment biopsies from 52 larynx cancer patients. Patients developing a local recurrence were matched for T-stage, subsite, treatment, gender and age with non-recurrence patients. Candidate genes were then tested by immunohistochemistry on tumor material from a second series of 76 patients. Both series comprised early stage cancer treated with radiotherapy alone. Finally, gene expression data of eight larynx cancer cell lines with known radiosensitivity were analyzed.

RESULTS:
Nineteen patients with a local recurrence were matched with 33 controls. Gene sets for hypoxia, proliferation and intrinsic radiosensitivity did not correlate with recurrence, whereas expression of the putative stem cell marker CD44 did. In a supervised analysis, probes for all three splice variants of CD44 on the array appeared in the top 10 most significantly correlated with local recurrence. Immunohistochemical analysis of CD44 expression on the independent validation series confirmed CD44's predictive potential. In 8 larynx cancer cell lines, CD44 gene expression did not correlate with intrinsic radiosensitivity although it did correlate significantly with plating efficiency, consistent with a relationship with stem cell content.

CONCLUSIONS:
CD44 was the only biological factor tested which significantly correlated with response to radiotherapy in early stage larynx cancer patients, both at the mRNA and protein levels. Further studies are needed to confirm this and to assess how general these findings are for other head and neck tumor stages and sites.

TRANSLATIONAL RELEVANCE:
Treatment choice for larynx cancer is based on clinical factors such as T-stage, but these are imprecise indicators of response. Having robust methods to predict outcome of a particular therapy would be extremely valuable, allowing a more rational treatment choice which should lead to greater tumor cell kill and also spare patients from toxic and ineffective therapies. Such predictors should include biological factors as well as clinical factors, given the heterogeneity in tumor biology even for patients presenting with similar sites and stages. The present study employed gene expression profiling in a series of larynx cancers and validated the result in a second similar series using immunohistochemistry. The principle predictor for outcome after radiotherapy was CD44, a putative stem cell marker. In addition, this study sheds light on potential mechanisms of radioresistance, which could lead to the design of targeted drugs for combining with radiation.

Introduction

The incidence of larynx cancer in the United States is around 4.5 cases per 100,000 per year (1). The 5-year relative survival percentage for localized disease has been stable at around 70-80% for the last 20 years (1). In early laryngeal cancer, radiotherapy is an effective treatment modality, with local control rates between 80-90% for T1 tumors (2). Partial laryngectomy or CO2 laser resection are alternative treatments with comparable survival rates, although when used as salvage after a failed radiotherapy course they have a higher complication rate (3). Treatment choice is mainly based on the estimated functional outcome and the preferences of the clinician. It would therefore be useful to predict beforehand which patients will benefit from radiotherapy. Prediction of resistance is also likely to be increasingly useful in the development of biological modifiers which increase the effects of radiation, providing an alternative treatment for resistant tumors.

Important clinical factors associated with local recurrence after radiotherapy are tumor stage, tumor size, radiotherapy fraction size and year of treatment (4). Treatment choice is now mainly based on T-stage (5), although this is still a relatively poor indicator of survival (6). Since clinical factors cannot provide an accurate prediction, it is likely that recurrence of a tumor can partly be explained by tumor biology. Three biological processes known to influence response to radiotherapy are intrinsic radiosensitivity (7), hypoxia (8) and repopulation (9). For each of these processes, individual markers (mainly immuno-histochemical) have been investigated and found to be of predictive value (10–12), although none have been sufficiently validated or are in routine use. Since many genes are involved in each process, in addition to single markers representing these processes, sets of markers (gene sets) for hypoxia (13, 14), intrinsic radiosensitivity (15–17) and repopulation (18) have also been defined. Another factor more recently hypothesized to play a role in response to therapy is the number of stem cells, ultimately determining repopulation of the tumor (19, 20) and so eradication of this subpopulation is of prime importance.

To date, no studies have investigated all these processes simultaneously. Microarrays have been used to measure gene expression (mRNA) on a genome wide scale, and can in principle monitor all the above-mentioned processes concurrently. However, only one microarray study with 14 patients has been carried out for patients treated with radiotherapy alone (21). Several expression profiling studies have been carried out on patients treated with radiotherapy in combination with surgery or chemotherapy (22–26). However, these have often included heterogeneous groups of patients and cannot address the question of factors affecting the response of laryngeal cancer to radiation alone.

Our objective was to find a gene expression profile that will accurately predict local recurrence after radiotherapy in a homogeneous group of patients with early laryngeal carcinoma. We chose to study early stage tumors, since these are likely to be more homogeneous than advanced tumors and also technically easier to treat, minimizing the chance of geographical misses. Treatment failure is then highly likely to be due to biological rather than technical factors. In addition to giving more insight into the molecular processes

underlying treatment failure, accurate prediction would enable treatment to be individualized, leading to increased survival and less unnecessary morbidity. We studied two series of early stage larynx cancer patients treated with radiotherapy alone. The first was a test series of frozen tumor specimens used to study global gene expression to discover predictive markers for local control, which were then validated on a second series by immunohistochemistry.

Materials and Methods

All studies reported here were done with approval of the local Medical Ethics Committees.

Gene expression series

Patients.
Fifty two patients were recruited from five different institutes in The Netherlands and were eligible if they had been treated for a T1 or T2 larynx carcinoma (Table 3.1), and pre-treatment fresh frozen tumor material was available. Patients were treated between 1997 and 2005, and staging was done either clinically or with a CT-scan. Because patients with small tumors did not have a CT-scan, tumor volumes could not be measured for the whole group. Treatment was radiotherapy alone with curative intent, applying fractionation schemes standard in each of the five centers. To compare different radiotherapy schedules, the equivalent dose in 2-Gy fractions (EQD_2) was calculated for every patient with the formula: $EQD_2 = D \times (d + \alpha/\beta)/(2 + \alpha/\beta)$, where D is the total dose, d the given fraction dose, the α/β ratio was assumed to be 10 Gy. Recurrence was defined as a histologically proven local tumor recurrence within two years of the initial treatment, to ensure the analysis of true recurrences rather than second primaries. Since we planned to study a matched series, for every patient with a recurrence we aimed to include two controls, with a recurrence-free follow-up of at least two years and matched for the institute they were treated in, T-stage, subsite, gender and age. There were no significant differences between groups with and without local recurrence in age, gender, subsite, T-stage, total dose, fraction size, tumor percentage or RNA quality (Table 3.1).

RNA isolation.
All biopsies were snap frozen in liquid nitrogen. Around 30 slices of 30 µm were deposited in RNA-Bee (Campro scientific). Before and after these 30 slices H&E sections were taken that were subsequently assessed by an experienced pathologist, who scored differentiation and tumor percentage. Only biopsies containing on average more than 50% of tumor cells were included. The tumor material in RNA-Bee was processed using the Qiagen RNeasy mini and RNase-free DNase kits. Total RNA was isolated and DNAse treated using spin columns according to the manufacturers instructions. The Agilent 2100 bioanalyzer was used to assess the integrity (intactness) of the RNA. Samples with an RNA Integrity Number (RIN) under 6.0 or with no obvious 18S and 28S peaks were discarded.

Baseline characteristics					
		No recurrence (N=33)		Recurrence (N=19)	
		N/ Average	%	N/ Average	%
Mean age at diagnosis (years)		63.5		63.7	
Gender	Male	26	78.8	17	89.5
	Female	7	21.2	2	10.5
Subsite	Supraglottic	11	33.3	3	15.8
	Glottic	21	63.6	16	84.2
	Subglottic	1	3.0	0	0.0
T-stage	T1	14	42.4	7	36.8
	T2	19	57.6	12	63.2
Center	Amsterdam	10	30.3	4	21.1
	Groningen	8	24.2	4	21.1
	Leiden	4	12.1	2	10.5
	Maastricht	3	9.1	4	21.1
	Nijmegen	8	24.2	5	26.3
Mean total dose (Gy)		66.5		66.3	
Mean fraction size (Gy)		2.1		2.1	
Mean treatment time (days)		39.6		39.0	
Mean EQD_2 (Gy)		67.0		66.8	
Mean tumor percentage		72.7		70.4	
Mean RIN		7.8		7.7	

Table 3.1. Baseline characteristics of patients and treatments in the expression profiling test series: The characteristics are shown separately for the groups with and without recurrences. EQD_2: dose recalculated to an equivalent dose in 2 Gy fractions. Tumor percentage: average percentage of tumor cells in the frozen biopsy used for RNA extraction. RIN: RNA integrity number.

Gene expression.

cDNA was made from one microgram of total RNA and amplified into aRNA with T7-mRNA Superscript-III amplification kit (Invitrogen). Only amplification yields over 1000-fold with a 1 kB smear on a gel were accepted. Hybridization to microarray slides was performed at our Central Microarray Facility (http://microarrays.nki.nl). All samples were hybridized to Illumina bead arrays (v3 Illumina beads) and subsequently scanned using the Illumina scanner. Each Illumina array consists of 3-micron silica beads covered with oligos containing over 48,000 transcript probes per sample, representing around 25,000 known genes. Each transcript probe was represented more than 20-fold per array and final data were averaged for each probe. Fluorescence intensities were measured with the Illumina scanner and averaged per probe.

Data analysis.
The dataset was transformed (variance stabilizing method (ref. 27)) and normalized (robust spline method) with the Lumi (28) package for R, version 2.8 (29) (http://www.R-project.org). If, for a specific probe, no patient had a value above background levels, that probe was filtered out. Gene sets for hypoxia, proliferation, radiosensitivity and stem cells were tested (13–16, 18, 30, 31). Unigene identifiers were used to map the genes in a set to the annotations of the Illumina array. For gene sets with known weights contributing to the endpoint (as described in the original publications), Pearson correlations were calculated against the weights of a gene set for each patient. This also allowed assessment of gene sets which included genes both positively and negatively correlating with outcome. For gene sets without weights (each gene assumed to contribute equally), the average expression of the genes in the set was calculated. For these signatures, all genes in the set were correlated in the same direction with outcome. The Pearson or average values were then used in a logistic regression with local recurrence data. In order to give comparable odds ratios, some Pearson correlations were multiplied by 5 or 10, which does not change the *P*-values but simply provides a better comparison of odds.

In addition to this hypothesis-driven analysis, a data-driven analysis was performed with Biometric Research Branch (BRB) array tools (NIH, http://linus.nci.nih.gov/brb-arraytools.htm). Genes were first filtered by including probes where at least 20% of samples had a minimum fold change greater than 1.35 and a *P*-value for log-ratio variation under 0.01. The filtered set was entered in a nearest centroid model that finds genes that best predict local recurrence. Genes significantly different between the patients with and without recurrence at the $P < 0.01$ significance level were used for class prediction. The leave-one-out cross-validation method was used to compute mis-classification rates.

Immunohistochemistry series

Patients.
Of the patients included in the mRNA expression microarray series, paraffin embedded material for immunohistochemistry (IHC) was used from two of the five institutes (Amsterdam and Groningen). This small subset of 20 cases was used to confirm gene expression values by IHC. A second matched series of 76 patients was used as an independent validation series of our findings from the gene expression study. Paraffin embedded biopsies were used to make cores for a tissue microarray (TMA). The construction of the TMA and the patient characteristics were described previously (10). Briefly, the patients were predominantly male with stage T1 and T2 glottic tumors given a median of 66 Gy in 2 Gy fractions (Table 3.2).

Immunohistochemistry staining.
Sections of 3 µm were cut from either whole tissue blocks or the TMA and mounted on amino-propyl-ethoxy-silan (APES, Sigma-Aldrich, Diesenhofen Germany)-coated glass slides. Slides were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval comprised boiling the slides in a microwave oven in citrate (pH 6.0) for 15 minutes. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase for 30 minutes. Slides were incubated with a mouse monoclonal antibodies against CD44 (156-3C11; dilution 1:200; Cell Signaling Technology, Danvers, MA) and CD44v6 (clone VFF-18; dilution 1:8000; Bender

Medsystems, Vienna, Austria) for 1 h at room temperature. Detection was performed with RAM^{HRP} (dilution 1:100) and GAR^{HRP} (dilution 1:100), visualized by 3'3-diaminobenzi-dinetetra-hydrochloride and counterstained with haematoxylin.

Baseline characteristics					
		No recurrence (N=47)		Recurrence (N=29)	
		N/ Average	%	N/ Average	%
Mean age at diagnosis (years)		65.9		59.4	
Gender	Male	39	83	27	93.1
	Female	8	17	2	6.9
Subsite	Supraglottic	21	44.7	4	13.8
	Glottic	25	53.2	25	86.2
	Unknown	1	2.1	0	0
T-stage	T1	8	17.0	11	37.9
	T2	28	59.6	13	44.8
	T3	6	12.8	5	17.2
	T4	5	10.6	0	0
Mean total dose (Gy)		68.9		65.4	

Table 3.2. Baseline characteristics of patients in the TMA validation series: The characteristics are shown separately for the groups with and without recurrences.

Immunohistochemistry scoring.

The percentage of tumor cells staining positive for CD44 was scored as well as the intensity of staining (low or high). A CD44 staining score was calculated by adding the percentages of positive low and high intensity cells, weighted by factor of 1 and 2 respectively. This weighted score reflects total CD44 protein better than total percentage positive cells, for better comparison with total mRNA from the microarray analysis. For the set of patients in which concordance between mRNA and IHC was tested, all slides were analyzed independently by two teams, each consisting of a pathologist (MvV and JvdW) and a scientist. Slides scored differently by the two teams were discussed at a conference microscope to reach consensus. Before consensus, the inter-observer correlation for CD44 scores was 0.75 ($P < 0.001$; Figure 3.1). For the TMA series of 76 patients, scoring was done by one team. Pearson correlations were calculated between mRNA levels and IHC scores. For the TMA analysis, associations between CD44 expression and local recurrence were compared using a logistic regression model. *P*-values of <0.05 were considered statistically significant. Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc., Chicago, IL).

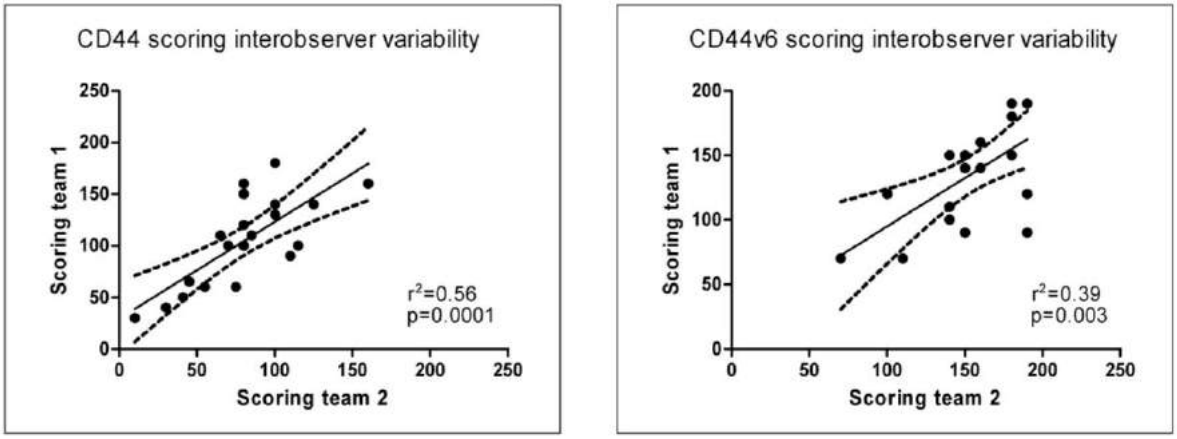

Figure 3.1. Interobserver variability between immunohistochemistry scores of the two teams for N=20.

Larynx cancer cell lines

Cell culture.

The larynx cancer cell lines UT-SCC-6A, -8, -9, -19A, -19B, -22, -23 and -42A from the University of Turku (Finland) were cultured in DMEM with 10% FBS, 1% NEAA, 1% L-glutamine and 1% penicillin-streptomycin. Information was available on plating efficiency and radiosensitivity for all cell lines (published and unpublished data) (refs. 32–34).

Gene expression.

For each cell line, 1×10^6 cells were washed with ice cold PBS at approximately 50% confluence and then collected in RNA-Bee. Illumina microarray data were generated using the same methods and materials as described above for the tumor biopsies.

Results

Gene expression

Gene expression analysis.

Exclusion of probes that did not exceed background expression in any patient left 26,454 probes for analysis. Gene expression signatures for hypoxia, intrinsic radiosensitivity, repopulation and stem cells were analyzed in a logistic regression (Table 3.3). The putative stem cell marker *CD44* was the most significant, with an unrelated stem cell signature as second most significant. A third stem cell signature not including *CD44* (Table 3.4) was fifth of the 12 signatures tested but was not significant. After multiple testing correction (Bonferroni), only *CD44* expression remained significant ($P = 0.024$). Comparative histograms of *CD44* expression illustrate the higher expression in recurrences versus cures (Figure 3.2A). When patients were divided into three groups of low, medium and high *CD44* expression, split so that there were equal numbers of recurrences in each group, the odds of recurrence (number of recurrences divided by number of non-recurrences for each group) was 15.2 fold higher in the highest *CD44* expression group compared with the lowest ($P = 0.003$, Figure

3.2B). Expression of acute hypoxia genes was also associated with local recurrence, although significance was lost after correction for multiple testing. Radiosensitivity and proliferation genes showed no relationship with recurrence.

Gene set	Range	p-value	OR	95% CI
Stem cell (CD44)(31)	7.8 - 9.1	0.002	20.2	3.4 – 172.3
Stem cell (Glinksky)(45)	5.7 - 6.9	0.03	6.5	1.3 - 42.2
Acute hypoxia (Chi) x10(14)	-1.6	0.04	7	1.1 - 46.6
Hypoxia metagene (Winter)(13)	7.2 - 7.9	0.13	19.3	0.5 – 1225.2
Stem cell genes (various) excluding CD44	6.4 - 7.4	0.16	7.5	0.4 – 129.7
Radiosensitivity (17), response	-0.5	0.64	0.3	0.002 – 46.0
Proliferation (Shepard*) x10	-1	0.67	1.6	0.2 - 12.2
Chronic hypoxia (14)	-1.6	0.67	0.7	0.1 - 3.8
Radiosensitivity (15)	6.1 - 7.2	0.68	0.6	0.03 - 9.4
Proliferation (18)	6.4 - 7.1	0.95	0.9	0.03 – 26.1

* From Gene Set Enrichment Analysis molecular signatures database; http://www.broadinstitute.org/gsea/msigdb/

*Table 3.3. Logistic regression of gene sets with local recurrence. Range: lowest to highest value of either Pearson correlations against the weights of a gene set or, for gene sets without weights, the average expression (log2 scale) of the genes in the set. OR: odds ratios with corresponding confidence intervals and p-values were generated from a logistic regression of Pearson or average values of the gene sets with local recurrence data. In order to give comparable odds ratios, some Pearson correlations were multiplied by 5 or 10 (not changing the p-values). *From Gene Set Enrichment Analysis molecular signatures database; http://www.broadinstitute.org/gsea/msigdb.*

Stem cell gene set		
ABCG5	ITGA6	MYC
ALDH1A1	ITGB1	PROM1
BMP4	ITGB3	REXO1
CD200	KLF4	SOX2
CD24	KRT15	TERC
CD34	KRT19	THY1
DPPA2	LGR5	TLE1
ITGA2	LRIG1	TNC

Table 3.4. Stem cell gene set, excluding CD44, gene symbols derived from various sources by the authors.

After restriction of the dataset to those 8,317 probes that showed significant differences in expression between the tumors, thus removing uninformative probes, we performed a data-driven analysis for which genes best predicted recurrence. When the univariate significance alpha level was set to $P < 0.01$, 34 probes (18 up and 16 down-regulated in tumors subsequently recurring) were found to be predictive (Table 3.5). The most significant upregulated marker discriminating between cures and recurrences was *CD44* ($P < 0.002$). With the nearest centroid method only 23% of the patients were correctly classified with these 34 genes. In addition, false discovery rates, as calculated by the Benjamini-Hochberg method, were high. However, despite the predictive weakness of the signature as a whole,

of note was that all three probes for *CD44* that were present on the array appeared in the top 10 highest ranking upregulated genes. Two of the probes (variants 4 and 5) map to the constant and largest exon (exon 18), while variant 1 maps to the first variable exon (exon 6). Expression of each probe was highly significantly correlated with expression of each of the other probes across the 52 tumors (all *P*-values <0.001; 1 vs 3, 1 vs 5, 3 vs 5).

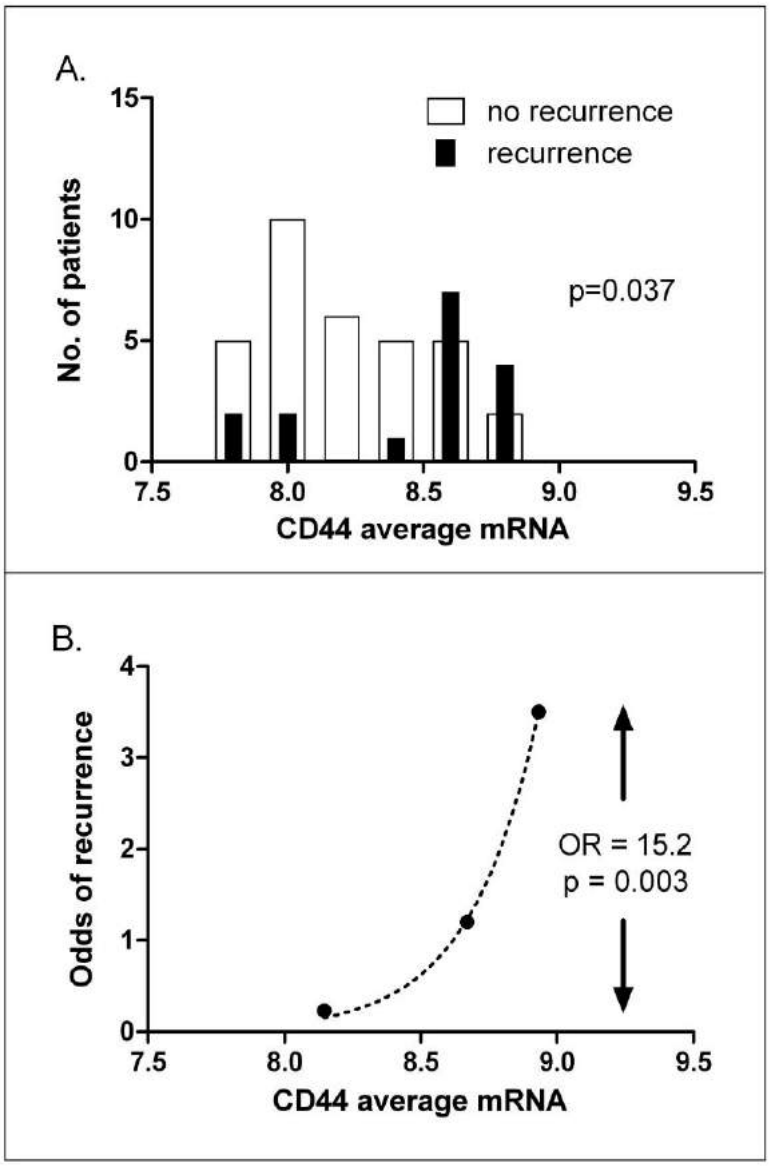

Figure 3.2. CD44 expression predicts local recurrence. A, histograms of CD44 mRNA expression for patients subsequently cured (open bars) or those subsequently suffering a recurrence (closed bars). B, odds of recurrence when patients are divided into three groups with increasing mRNA levels, split so that each group contains equal numbers of recurrences. OR: odds ratio of recurrence between highest and lowest CD44 expression groups.

Gene symbol	t-value	Parametric p-value	Probe ID	Description
UP-REGULATED in recurrence				
CD44	-3.485	0.001	ILMN_1803429	CD44 molecule (Indian blood group), transcript variant 4
HSD17B12	-3.170	0.003	ILMN_1702168	hydroxysteroid (17-beta) dehydrogenase 12
BTBD11	-3.086	0.003	ILMN_1705066	BTB (POZ) domain containing 11, transcript variant 1
CHL1	-3.077	0.003	ILMN_1713347	cell adhesion molecule with homology to L1CAM
MGLL	-3.000	0.004	ILMN_1738589	monoglyceride lipase, transcript variant 1
BNIP3	-2.925	0.005	ILMN_1724658	BCL2/adenovirus E1B 19kDa interacting protein 3
SNX5	-2.921	0.005	ILMN_1673676	sorting nexin 5, transcript variant 1
CD44	-2.851	0.006	ILMN_1778625	CD44 antigen (Indian blood group), transcript variant 1
CAPRIN1	-2.823	0.007	ILMN_1754145	cell cycle associated protein 1, transcript variant 1
CD44	-2.803	0.007	ILMN_2348788	CD44 molecule (Indian blood group), transcript variant 5
CHMP2A	-2.775	0.008	ILMN_1656621	chromatin modifying protein 2A, transcript variant 1
SLC37A4	-2.748	0.008	ILMN_1678678	solute carrier family 37 (glucose-6-phosphate transporter), member 4
CNDP2	-2.724	0.009	ILMN_1726769	CNDP dipeptidase 2 (metallopeptidase M20 family)
ATP1B1	-2.706	0.009	ILMN_1730291	ATPase, Na+/K+ transporting, beta 1 polypeptide, transcript variant 1
HK1	-2.700	0.009	ILMN_1761829	hexokinase 1, transcript variant 1
ACADVL	-2.696	0.009	ILMN_2263466	acyl-Coenzyme A dehydrogenase, very long chain, transcript variant 1
IL1B	-2.694	0.009	ILMN_1775501	interleukin 1, beta
TLE1	-2.691	0.010	ILMN_1751572	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)
DOWN-REGULATED in recurrence				
PRSS21	2.704	0.009	ILMN_1774256	protease, serine, 21 (testisin), transcript variant 2
AGPAT4	2.705	0.009	ILMN_1730504	1-acylglycerol-3-phosphate O-acyltransferase 4
RNF7	2.707	0.009	ILMN_1711862	ring finger protein 7, transcript variant 3
LSM1	2.754	0.008	ILMN_2218450	LSM1 homolog, U6 small nuclear RNA associated (S. cerevisiae)
GPATCH2	2.755	0.008	ILMN_1786036	G patch domain containing 2
GOLGA7	2.794	0.007	ILMN_1778673	golgi autoantigen, golgin subfamily a, 7, transcript variant 2
C3orf21	2.831	0.007	ILMN_1671116	chromosome 3 open reading frame 21
TLOC1	2.841	0.006	ILMN_1762003	translocation protein 1
HIST2H2AC	2.858	0.006	ILMN_1768973	histone cluster 2, H2ac
BRF2	2.885	0.006	ILMN_1665554	subunit of RNA polymerase III transcription initiation factor, BRF1-like
SLMO1	2.904	0.005	ILMN_2232157	slowmo homolog 1 (Drosophila)
LYPLAL1	3.053	0.004	ILMN_2142117	lysophospholipase-like 1
MRPL55	3.074	0.003	ILMN_2348090	mitochondrial ribosomal protein L55, transcript variant 5
TERC	3.099	0.003	ILMN_1766573	telomerase RNA component on chromosome 3
MRPS1	3.212	0.002	ILMN_1663664	mitochondrial ribosomal protein S10
KIAA97	3.508	0.001	ILMN_1670752	KIAA0907

Table 3.5. Data driven classifier, showing top 34 most significant genes, all with a p-value <0.01.

In addition to *CD44*, the remaining top ranking genes from Table 3.5 were most highly represented in a pathway relevant to "cell cycle, cellular development, cellular growth and proliferation" (from Ingenuity Pathway Analysis). This pathway contained *EGF, VEGF,* and *HRAS* as hub genes and of 35 genes on the pathway, 11 appeared in list of top ranking genes (Figure 3.3).

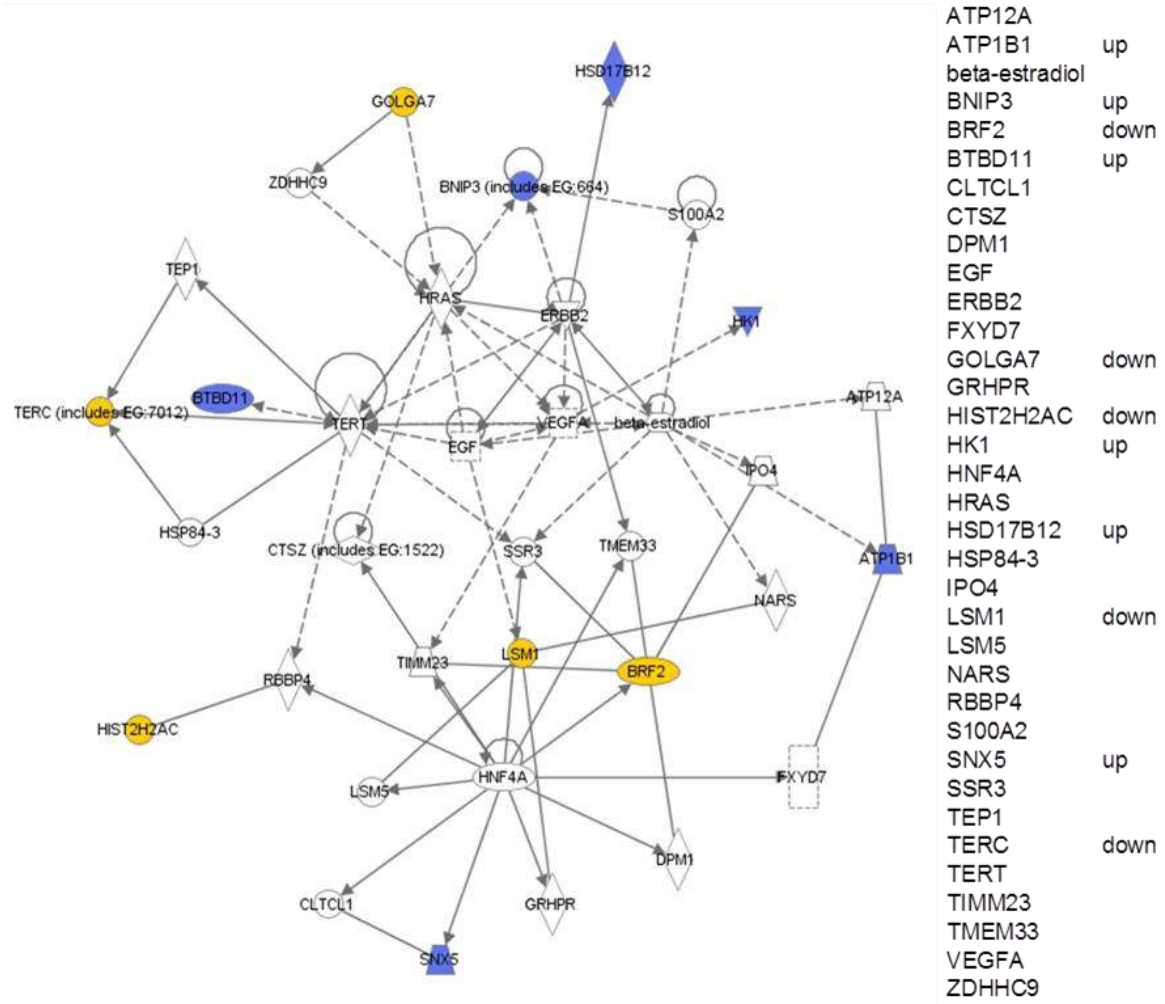

Figure 3.3. Highest ranking pathway from Ingenuity Pathway Analysis of the top 34 genes arising out of the data-driven analysis of cures versus recurrences (see table 3.5). Blue: up regulated in recurrences; yellow: downregulated in recurrences. For clarity, all genes are also listed on the right, indicating whether they are up or down regulated.

CD44 protein level versus outcome

CD44 mRNA correlates with immunohistochemical expression.

Both frozen and paraffin embedded material was readily available from 20 tumors and used to compare RNA and protein expression. Antibodies were tested against an epitope common to all CD44 variants and one specific for the v6 variant. Figure 3.4 shows examples of CD44 staining. All tumors showed some expression (with on average 24% of tumor cells staining with a low intensity and 52% with a high intensity) although the staining was heterogeneous in all cases. In tumors showing a clear differentiation pattern, the basal cell layers were more intensely stained than the more differentiated cells. Both the CD44 and the CD44v6 immunostaining scores correlated significantly ($P < 0.05$) with the average for all three CD44 mRNA probe levels (Figure 3.5).

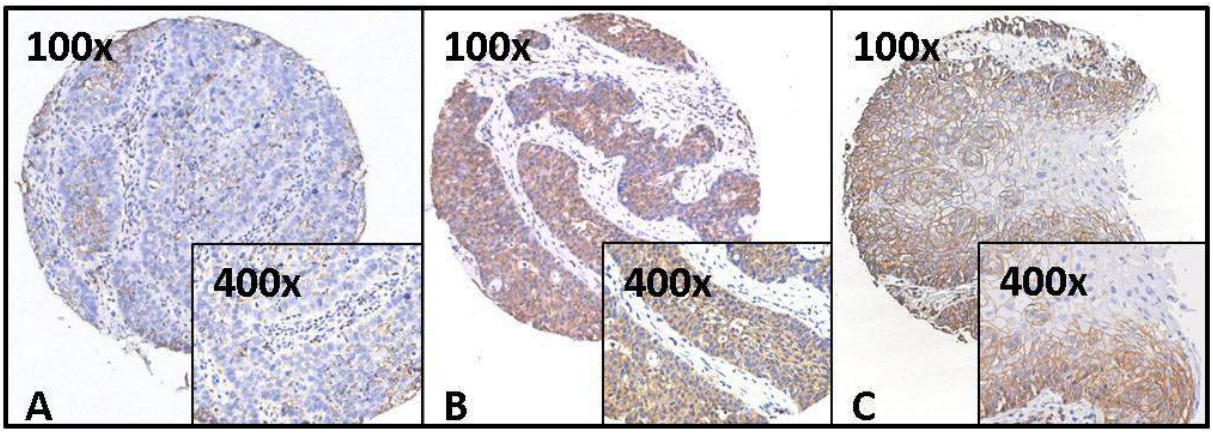

Figure 3.4. Examples of CD44 immunohistochemistry. Staining, using antibody 156-3C11, against an epitope common to all CD44 variants, on the tissue microarray for three representative cores at two different magnifications (100× and 400×). Scorings for these cores were: A: 40% intensity I. B: 95% intensity II. C: 80% intensity II.

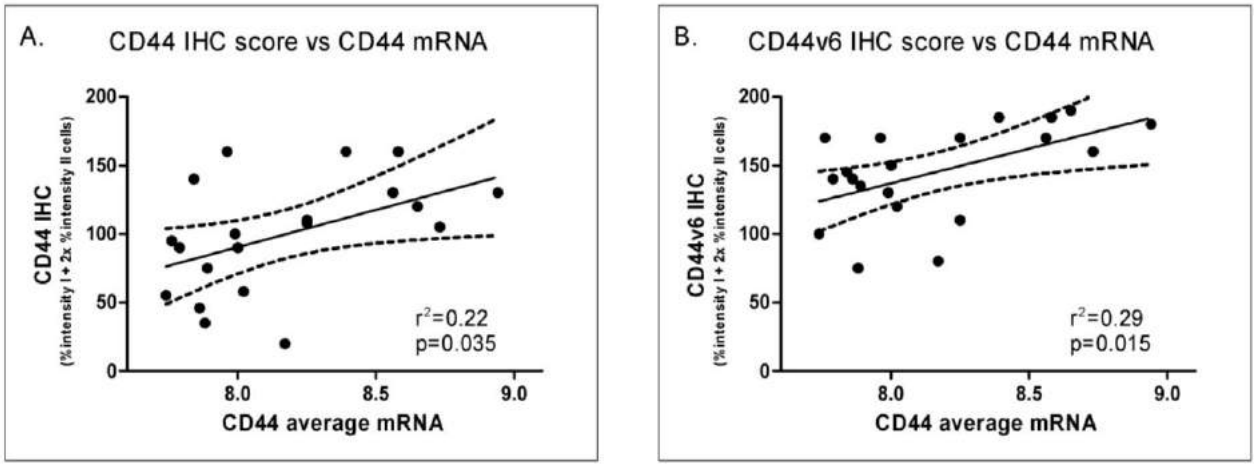

Figure 3.5. Correlation between mRNA expression and immunohistochemistry for CD44 (A) and CD44v6 staining (B). IHC: immunohistochemistry.

CD44 expression in the validation series.

We next tested whether immunohistochemical expression of CD44 correlated with clinical outcome. We used an independent matched series of laryngeal cancers with patient characteristics similar to the test series. Patient characteristics of this validation series, like the 52 patients in the test series, were predominantly male with a T1-2 glottic tumor and treated with radiotherapy alone (Table 3.2). CD44 expression, assessed immuno-histochemically for percentage CD44-positive cells weighted according to staining intensity (see Materials and Methods), was significantly associated with clinical outcome. Histograms of the IHC scores showed higher CD44 protein expression in recurrences compared with cures (Figure 3.6A). As before, when patients were divided into three groups with low, medium and high CD44 expression, split to ensure equal numbers of recurrences per group, the odds ratio for recurrence was 6.1 fold higher in the highest group compared with the lowest (P = 0.005, Figure 3.6B). These data on protein expression thus confirm the mRNA expression data.

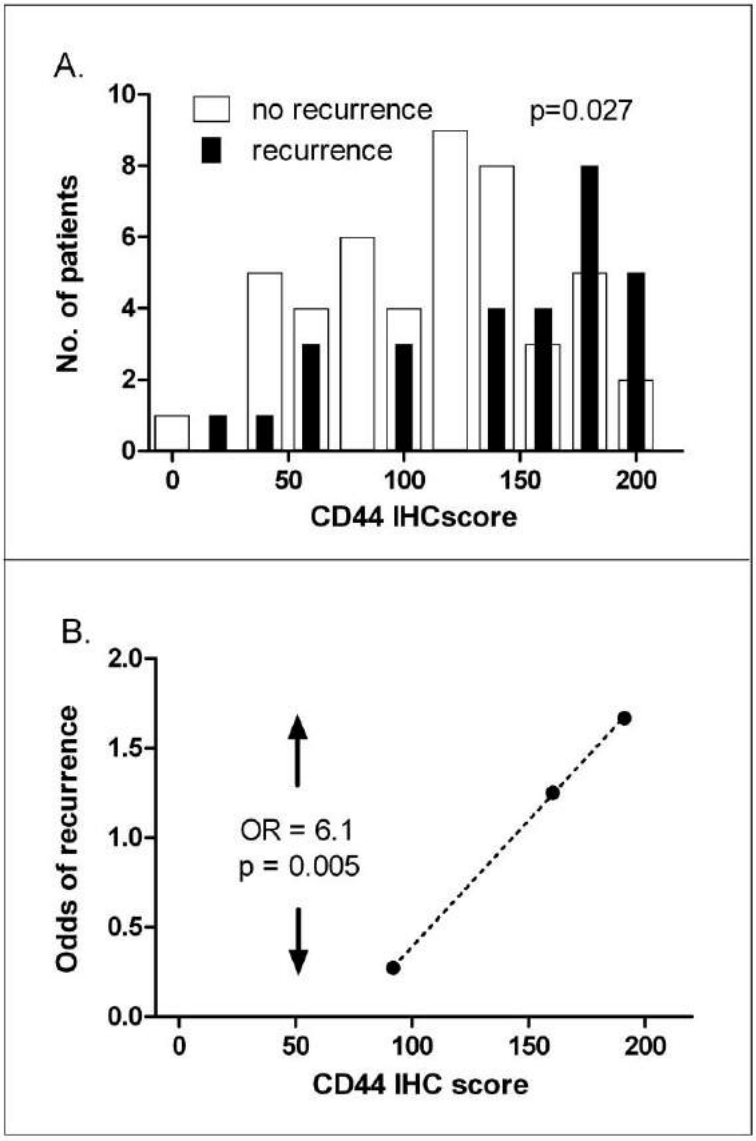

Figure 3.6. CD44 IHC predicts local recurrence. A, histograms of CD44 IHC score for patients subsequently cured (open bars) or those subsequently suffering a recurrence (closed bars). B, odds of recurrence when patients are divided into three groups with increasing IHC scores, split so that each group contains equal numbers of recurrences. OR: odds ratio of recurrence between highest and lowest CD44 expression groups.

Larynx cancer cell lines

In addition to cellular radiosensitivity, the effectiveness of fractionated radiotherapy can be determined by microenvironmental factors such as hypoxia, repopulation rates during therapy, and the fraction of stem cells. As a first step in attempting to dissect the role played by *CD44* on these factors, we studied a series of larynx cancer cell lines under well controlled *in vitro* conditions. As shown in Figure 3.7, *CD44* mRNA levels (average for the three probes) correlated significantly with plating efficiency (P = 0.03). Since plating efficiency has been correlated with tumor initiating capacity in several studies, this is consistent with *CD44* being a stem cell marker in this tumor type. In the same experiments, CD44 expression did not correlate with intrinsic radiosensitivity in these 9 larynx cancer cell lines (P-value = 0.71).

None of the three *CD44* probes individually showed a correlation with radiosensitivity, while two out of three CD44 probes show a significant correlation with plating efficiency (Table 3.6). These data imply that *CD44* expression is not monitoring intrinsic radiosensitivity but rather the fraction of stem cells.

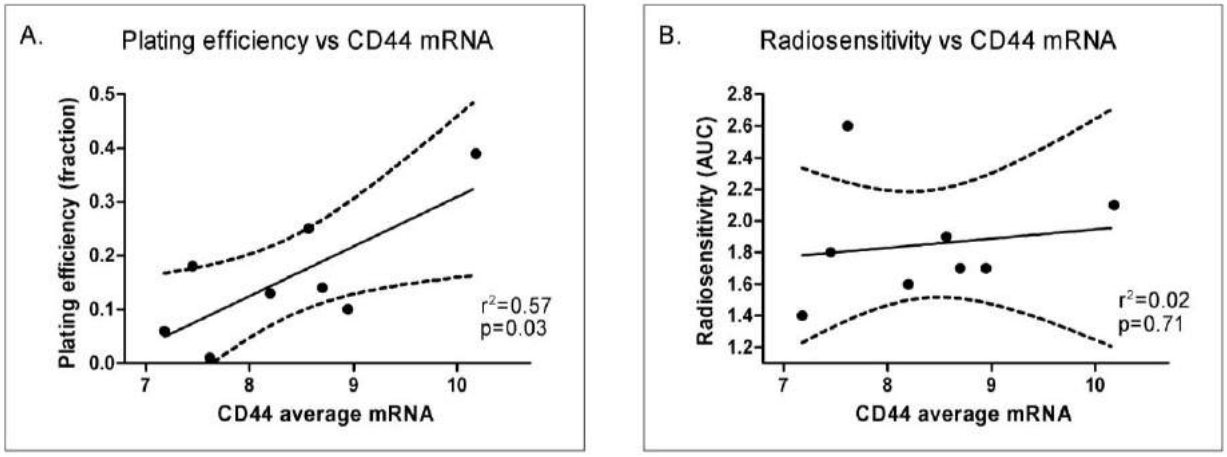

Figure 3.7. Correlation of plating efficiency (A) and radiosensitivity (B; as measured by area under the survival curve (AUC)) with CD44 mRNA levels (averaged over the 3 probes).

CD44 mRNA in cell lines			
CD44 mRNA		Pearson corr. with	
		PE	AUC
Probe	v1	0.61	0.05
	v4	0.71*	0.12
	v5	0.80*	0.23
Average		0.76*	0.15

*Table 3.6. Correlation of CD44 expression with plating efficiency (PE) and radiosensitivity (area under radiation survival curves, AUC). V1, v4 and v5 are three separate probes for CD44 mapping to exons 6, 18 and 18 respectively. *Significant at the 0.05 level (2-tailed).*

Discussion

The aim of this study was to find prediction markers for clinical outcome of larynx cancer after radiotherapy using gene expression profiling. We chose to study early stage tumors since these will be inherently less variable than advanced cancer both in terms of genetically different subpopulations and variability in blood flow and hypoxia. In addition, delivery of the radiotherapy is less complicated with less chance of geographical misses. Any recurrences are therefore likely to be due to inherent resistance of the tumor cells. Secondly, we chose to match recurrent and non-recurrent patients for the most important

known clinical variables (T-stage, subsite, treatment, gender and age), so that these would not be confounding factors in the analysis.

In the test series, we studied the expression of several sets of genes monitoring biological processes known to influence the outcome of radiotherapy. We found that *CD44*, chosen as a stem cell marker, showed the most significant correlation with local recurrence. Expression of genes monitoring proliferation and intrinsic radiosensitivity showed no correlation with outcome. A gene set defining acute hypoxia showed a trend, although not significant when corrected for multiple testing. In a separate data-driven analysis including over 8000 genes (after filtering out genes not showing significant expression or significant variation across the samples), the three probes for *CD44* came out high in the ranking list of genes correlating with recurrence, one of the probes being the most significant of all genes tested. This non-hypothesis-driven approach supported the hypothesis-driven approach, indicating that *CD44* is a good predictor of outcome after radiotherapy in these head and neck squamous cell carcinomas. Furthermore, in an independent validation series, CD44 protein expression measured immunohistochemically correlated significantly with outcome, such that higher CD44 scores were associated with a higher chance of local recurrence. Since both these were matched series, results are independent of the most important clinical predictors.

In a previous expression profiling study from our own institute on a series of 91 HNSCC patients treated with concurrent radiation and cisplatin, *CD44* was higher in tumors from patients which subsequently developed a recurrence, although this did not reach significance (P = 0.08) (ref. 25). Kawano et al found CD44s and CD44v6 staining correlated with prognosis in a series of 57 patients treated with surgery and radiotherapy (35). Zhao et al analyzed margins after surgery for 112 HNSCC patients and found that CD44v6 presence in these margins, detected with immunohistochemistry, was predictive of recurrence (36). Wang et al. (37) found that one CD44 isoform (v10) was associated with reduced disease free survival in HNSCC. These, together with the present study, support CD44 expression as a negative predictive factor.

We chose *CD44* as a stem cell marker for HNSCC, since Prince et al. (31) showed that CD44 positive cells in this tumor type were up to an order of magnitude more tumorigenic than CD44 negative cells. These data indicated that CD44 positive cells are enriched in cancer stem cells. However, our and other (38) IHC studies showed a relatively high average percentage of cells staining for CD44, inconsistent with a small minority stem cell fraction. We and others also observed a gradient of CD44 staining, where cells in more basal-like areas stained more positively than cells in the more differentiated areas. Such patterns may reflect more stem like properties of cells in the basal-like areas, analogous to that in normal epithelia.

Assuming that *CD44* has a causal role in determining the chance of recurrence and is not simply an indirect marker for stem cell content or another unknown process, there are several possible explanations for this role in the many functions of CD44. CD44 is a transmembrane glycoprotein with many transcript variants and has hyaluronan, an extracellular matrix protein, as a ligand (39). Various functions of CD44 have been described, including promoting tumorigenesis, cell motility and invasion. CD44, when activated by ligand, can act as a co-receptor for several membrane receptors, triggering various

intracellular signalling pathways. In one of these, CD44 acts as co-receptor for the ErbB family which can lead to activation of the *PI3K/AKT* pathway, a pathway known to promote survival after cytotoxic damage, including after irradiation. This suggests a possible link between *CD44* expression and intrinsic radiosensitivity. However, we did not find a correlation between *CD44* expression and radiosensitivity in the panel of larynx cancer cell lines. Alternatives therefore need to be sought to explain the relationship between *CD44* expression and radiocurability.

Other possibilities are links with hypoxia or repopulating ability, both known to influence radiotherapy outcome. We found that *CD44* expression correlated with expression of acute hypoxia genes (Figure 3.8) and a trend (P = 0.08) that expression of acute hypoxia genes correlated with chance of recurrence. No significant relationship with expression of chronic hypoxia genes was found. This is consistent with other studies indicating that cells hypoxic for relatively short times are more dangerous than those chronically exposed to hypoxia (40, 41). We found no evidence of a link between *CD44* expression and proliferation associated genes, or in this series between expression of proliferation genes and outcome. This is consistent with our earlier expression profiling studies on advanced head and neck tumors treated with radiotherapy and cisplatin, where proliferation genes were not predictive (25). Whether this is due to relatively slow repopulation rates in these tumors, or because the signatures do not adequately monitor repopulation capacity during fractioned radiotherapy is not known.

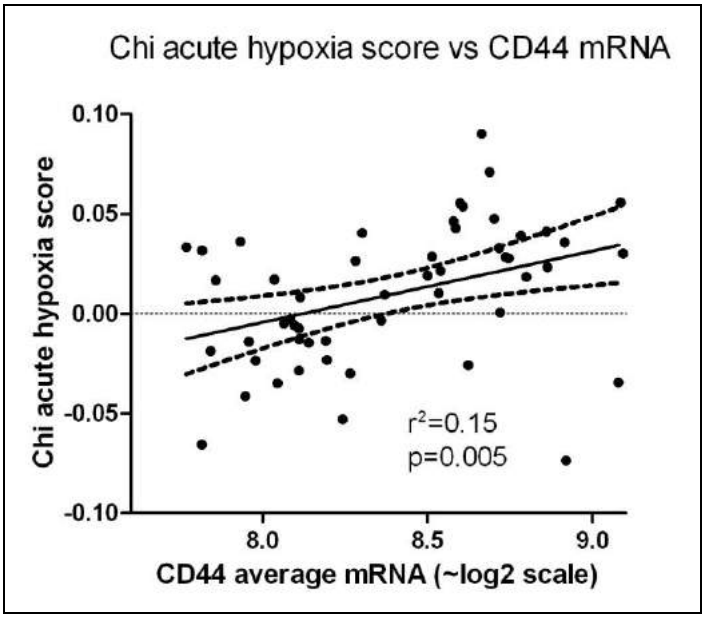

Figure 3.8. CD44 gene expression, averaged for the three probes, versus acute hypoxia signature score from Chi et al[14].

A final possible explanation is that *CD44* expression monitors the number of stem or cancer initiating cells. It is unlikely that all CD44-positive cells have stem cell properties, considering the rather ubiquitous expression of *CD44* in normal tissues (www.genecards.org), and the relatively high average fraction of CD44-positive cells in the tumors studied here and elsewhere (37, 38). However, if the cancer stem cells are a constant subfraction of CD44-positive tumor cells, the stem cell fraction (or tumor initiating fraction) will be directly

correlated with the CD44-positive fraction. In the current study, this fraction varied by a factor of around 3. Based on Poisson statistics, such a three-fold change in the effective number of cells which need to be killed by radiation would lead to an absolute change in the cure probability of around 30%; e.g. 1 surviving cell on average would lead to 37% cure probability, whereas 3 surviving cells on average would lead to a 5% cure probability. It is therefore possible that the relationship between cure and CD44 expression is a reflection of the number of cancer initiating cells needed to be killed. This is independent of whether the putative stem cells are more or less radioresistant than bulk tumor cells.

This contention is supported by the cell line data where *CD44* expression correlated significantly with colony forming efficiency of unirradiated cells (and not with radiosensitivity). This suggests a correlation with cancer initiating properties, since several studies have shown a correlation between *in vitro* plating efficiency and the number of cells required to produce tumors in animals (42–44). In addition, the Glinsky signature (45), a putative stem cell signature, also showed a strong trend with outcome in the test series (Table 3.3). This *BMI-1*-driven signature was derived by comparing primary and metastatic prostate cancer. We performed an Ingenuity pathway analysis on this 11-gene signature, also including CD44. The only significant pathway resulting from the analysis showed a link between the Glinsky genes and CD44 through an interaction with TGFB1 (Figure 3.9). While not definitive, these data support the notion that *CD44* is in some way monitoring stem cell capacity.

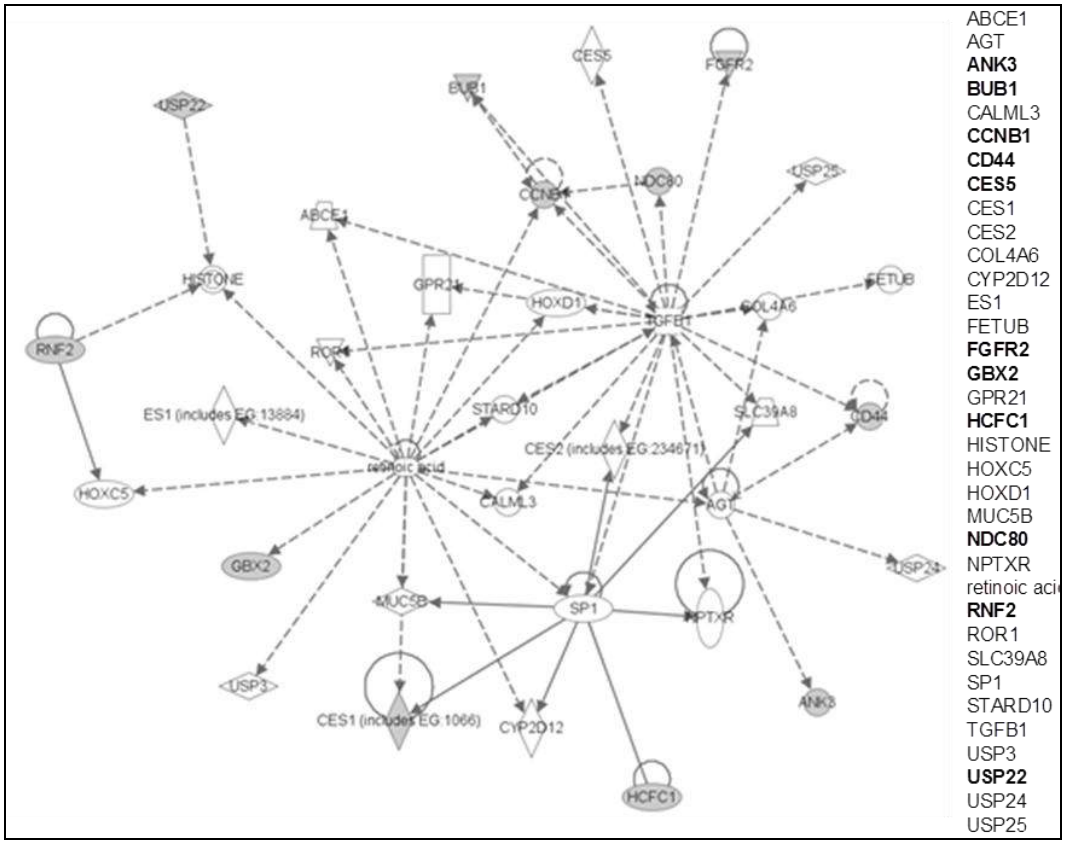

Figure 3.9 Link between CD44 and the Glinsky signature genes. The figure shows the only significant pathway arising from an Ingenuity Pathway Analysis where the input gene list was CD44 plus the 11-gene Glinsky signature. For clarity, all genes are also listed on the right:

genes in bold indicate those occurring on the input list. It can be seen that CD44 is linked indirectly to the Glinsky genes via interactions with TGFB1 and AGT.

Various CD44 isoforms have been described with different functions (39). In the present study, correlations with outcome were found with mRNA probes for one of the constant regions, and with an antibody against a constantly expressed epitope. Whether variant isoform expression would provide better prediction or understanding of failure needs further study.

Summary and Conclusion

CD44 expression, both at the mRNA and protein levels in independent patient series, correlated with the probability of recurrence after radiotherapy for early stage larynx cancer. Possible explanations are that *CD44* expression monitors the cancer stem cell fraction or that *CD44* expression monitors the hypoxic fraction. It will be important to distinguish these two possibilities, since interventions to increase cure in patients with high *CD44* expressing tumors will depend on the mechanism (attacking hypoxia, or attacking CD44 itself, or its downstream pathways, or other stem cell specific pathways). Predicting outcome is important partly to spare patients ineffective and toxic therapies. It will be equally or more valuable to provide alternative therapies for patients with resistant tumors. It is likely that CD44 expression, measured with standard immunohistochemical or perhaps PCR-based assays will contribute to better outcome prediction, and the next steps will be to confirm mechanisms and design effective interventions against the consequences of this over-expression. The present data suggest that the association between *CD44* and radioresponse reflects an increased number of cancer initiating cells that are usually resistant to radiation and result in a recurrence. *CD44* might therefore provide a new marker to predict the radiotherapy response in a biopsy of the primary tumor before treatment is initiated.

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CHAPTER 4

Pretreatment microRNA expression impacting on epithelial-to-mesenchymal transition predicts intrinsic radiosensitivity in head and neck cancer cell lines and patients

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Abstract

PURPOSE

Predominant causes of head and neck cancer recurrence after radiotherapy are rapid repopulation, hypoxia, fraction of cancer stem cells, and intrinsic radioresistance. Currently, intrinsic radioresistance can only be assessed by *ex vivo* colony assays. Besides being time-consuming, colony assays do not identify causes of intrinsic resistance. We aimed to identify a biomarker for intrinsic radioresistance to be used before start of treatment and to reveal biologic processes that could be targeted to overcome intrinsic resistance.

EXPERIMENTAL DESIGN

We analyzed both microRNA and mRNA expression in a large panel of head and neck squamous cell carcinoma (HNSCC) cell lines. Expression was measured on both irradiated and unirradiated samples. Results were validated using modified cell lines and a series of patients with laryngeal cancer.

RESULTS

miRs, mRNAs, and gene sets that correlated with resistance could be identified from expression data of unirradiated cells. The presence of epithelial-to-mesenchymal transition (EMT) and low expression of miRs involved in the inhibition of EMT were important radioresistance determinants. This finding was validated in two independent cell line pairs, in which the induction of EMT reduced radiosensitivity. Moreover, low expression of the most important miR (miR-203) was shown to correlate with local disease recurrence after radiotherapy in a series of patients with laryngeal cancer.

CONCLUSIONS

These findings indicate that EMT and low expression of EMT-inhibiting miRs, especially miR-203, measured in pretreatment material, causes intrinsic radioresistance of HNSCC, which could enable identification and treatment modification of radioresistant tumors.

TRANSLATIONAL RELEVANCE

In head and neck squamous cell carcinomas (HNSCC), radiation is a major treatment modality. Intrinsic radioresistance of tumor cells is one of the predominant causes of head and neck cancer recurrence. This phenomenon can only be examined by *ex vivo* colony assays, but these take too much time to be clinically useful and do not reveal the biologic mechanisms of intrinsic radioresistance. Using microRNA and mRNA expression profiles of HNSCC cell lines and tumors, we found that low expression of certain microRNAs that suppress epithelial-to-mesenchymal transition, measured prior to treatment, is causally related to intrinsic resistance to radiation. This finding provides an important step toward modification and thereby improvement of the treatment of radioresistant tumors.

 Introduction

Radioresistance of head and neck cancer

Radiotherapy is the most important treatment modality in head and neck cancer, with two thirds of patients treated with (chemo-)radiotherapy (1). With altered fractionated radiotherapy, the locoregional control rates for earlier stages are encouraging, but for stage III and IV tumors, locoregional control remains around 50% (2), leaving considerable need for improvement. Factors that contribute to control of the tumor are tumor site, stage, treatment schedule and dose, tumor volume, and HPV status (3–5). However, even after correcting for these factors, there are still differences in control rates. Such differences may result from differences in tumor microenvironment, tumor cell properties like hypoxia, rapid repopulation between fractions, the fraction of cancer stem cells or intrinsic radiosensitivity (6).

Intrinsic or cellular radiosensitivity is a term used to describe the process of one tumor cell being more resistant than another on the basis of different intracellular mechanisms, independent of microenvironmental factors.

An appropriate way to study intrinsic radiosensitivity is therefore in tissue culture in which potential confounding factors can be reduced or eliminated. It has indeed been shown that intrinsic cellular radiosensitivity significantly determines the outcome of radiotherapy in head and neck cancer (7). However, these data were attained using functional (cell survival) studies, giving limited or no information on genes or pathways involved and thus providing little help to the treating physician on how to improve treatment for patients with radioresistant tumors. We therefore searched for genetic and thus potentially assessable and targetable factors that affect intrinsic radioresistance in head and neck cancer.

mRNA to study radioresistance

mRNA profiling has been used to study radioresistance in cell lines. To date, however, such experiments have been mostly performed on either one or two cell lines only, or on the NCI-60 cell line panel, which contains no head and neck squamous cell carcinoma (HNSCC) lines (8, 9). Because it is known that radiosensitivity is partly dependent on the tissue of origin (e.g., lymphomas are more sensitive than solid tumors), use of such a cell line panel to predict HNSCC radiosensitivity is of questionable value. Therefore, Hall and colleagues attempted to identify a robust gene signature associated with intrinsic radiosensitivity on a series containing 16 cervical and 11 HNSCC cell lines. Unfortunately, they failed to identify such a set (10). Possibly this could be attributed to the fact that mRNA levels alone give an incomplete picture of active processes in the cell, as other factors can influence translation to protein. Among these are microRNAs (miR).

microRNAs

miRs are genomically encoded small pieces of single-stranded RNA of around 22 nucleotides each of which can silence hundreds of genes (11). More than 1,000 miRs have been identified so far,

estimated to regulate expression of at least 60% of all genes (12). miRs regulate gene expression by binding to their (partly) complementary sequence on mRNA molecules, resulting in reduced protein production (13, 14). miRs can reduce protein production by causing degradation of mRNAs or by inhibiting translation. Multiple modes of silencing thus seem to exist that can be active concurrently (15, 16).
Ionizing radiation has been shown to induce significant changes in miR expression in 6 cancer cell lines (17). miRs playing a role in radioresistance have been described, although experiments were done in cell line pairs and not in a larger panel of cell lines (18–20).

Study goal

The goal of this study was therefore to get a better insight into the genetic causes of intrinsic radioresistance in head and neck cancer cells focusing on miR expression. Using a large panel of HNSCC cell lines, we aimed to answer the following questions: (i) Do miR/mRNA expression changes induced by irradiation correlate with radioresistance?; (ii) Can we identify mRNAs that correlate with radioresistance?; (iii) Can we identify driving miRs that correlate with radioresistance?; (iv) If so, are these miRs and their targets related to certain pathways or processes?; and (v) Finally, do these miRs correlate with radiotherapy response in patients with laryngeal cancer? The answers to these questions should lead to a better understanding of radioresistance in this disease and therefore provide guidance toward more individualized treatment.

Materials and methods

Cell line selection and culture

Cell line selection.
All cell lines for hypothesis generation were obtained from Professor R. Grénman (University of Turku, Turku, Finland), who has a unique panel of more than 100 well-characterized HNSCC cell lines with known radiosensitivity. We selected 32 HNSCC cell lines from different subsites (Table 4.1). Cell lines previously treated with chemotherapy or derived from metastatic sites other than regional lymph nodes were excluded.

Cell culture.
All cells were cultured in DMEM, supplemented with 1% l-glutamine, 1% nonessential amino acids, 10% FBS, and antibiotics. Cells were incubated in humidified air with 5% CO_2 at 37°C. Depending on the doubling time, cells were subcultured every 3 to 14 days to ensure exponential growth. Cells were used for experiments when they were around 60% to 70% confluent. Preferably, low passages (10–20) were used.

Cell line	Radiosens-itivity (AUC)	Passage tested	Patient sex	Primary location	T	N	M	Type of specimen	Grade	Previous treatment
UT-SCC-1A	1.7	19	F	gingiva mandibulae	2	1	0	rT	2	RT
UT-SCC-2	1.8	12	M	floor of mouth	4	1	0	pT	2	no
UT-SCC-4	1.7	9	F	supraglottic	3	0	0	rN	2	RT
UT-SCC-5	2.3	14	M	tongue	1	1	0	ppT	2	RT
UT-SCC-6A	2.6	27	F	supraglottic	2	1	0	rT	1	RT
UT-SCC-7	2	12	M	cutis regio temporalis	1	0	0	rN	2	RT
UT-SCC-8	1.9	27	M	supraglottic	2	0	0	pT	1	no
UT-SCC-9	1.4	13	M	glottic larynx	2	1	0	N	1	RT
UT-SCC-12	2.1	14	F	cutis nasi	2	0	0	pT	1	no
UT-SCC-15	2.1	15	M	tongue	1	0	0	rT	1	RT
UT-SCC-16A	1.8	17	F	tongue	3	0	0	pT	3	RT
UT-SCC-19A	1.7	14	M	glottic larynx	4	0	0	pT	2	no
UT-SCC-19B	1.7	14	M	glottic larynx	4	0	0	ppT	2	RT
UT-SCC-20A	2.1	19	F	floor of mouth	1	0	0	pT	2	RT
UT-SCC-22	1.8	25	M	glottic larynx	1	0	0	rT	2	RT
UT-SCC-23	1.6	22	M	glottic larynx	3	0	0	ppT	1	RT
UT-SCC-24A	2.6	24	M	tongue	2	0	0	pT	2	no
UT-SCC-25	2.2	12	M	tongue	2	0	0	pT	1	RT
UT-SCC-27	1.9	12	M	gingiva mandibulae	2	0	0	rT	3	RT
UT-SCC-32	1.7	16	M	tongue	3	0	0	ppT	1	RT
UT-SCC-36	2.2	8	M	floor of mouth	4	1	0	pT	3	no
UT-SCC-42A	2.1	7	M	supraglottic	4	3	0	pT	3	no
UT-SCC-45	2	17	M	floor of mouth	3	1	0	pT	3	no
UT-SCC-46A	1.6	11	M	gingiva maxillae	1	0	0	pT	3	no
UT-SCC-47	2	13	M	floor of mouth	2	0	0	pT	3	no
UT-SCC-48	1.6	15	M	parotid gland	3	0	0	pT	2	no
UT-SCC-54C	2.3	14	F	buccal mucosa	0	0	0	rN	0	RT
UT-SCC-60B	2.2	13	M	tonsil	4	1	0	ppN	1	RT
UT-SCC-76A	2.5	13	M	tongue	3	0	0	pT	2	no
UT-SCC-77	2.5	23	M	tongue	1	0	0	rN	2	no
UT-SCC-79A	2.4	14	F	parotid gland	2	0	0	rT	2	no
UT-SCC-90	2.2	20	M	tongue	1	0	0	rT	2	RT

Table 4.1. Overview of the properties of all 32 cell lines. p= primary tumor, r= recurrent tumor, pp = persistent primary tumor, T=from the primary tumor location, N = from the lymph node.

Validation cell lines.
The UT-SCC-43A and UT-SCC-43A-Snail cell lines were developed and provided by Dr M. Takkunen (University of Helsinki, Helsinki, Finland; ref. 21). The FaDu-cDNA3 and FADU-HIF1α(ΔODD) cell lines were developed and provided by Prof. Kou-Juey Wu (National Yang-Ming University, Taiwan, ROC; ref. 22). Both cell lines are human HNSCC, transfected with either the transcription factor snail or HIF1α with a deleted oxygen degradation domain, thereby causing the cells to undergo epithelial-to-mesenchymal transition (EMT).

Irradiation assay

Radiosensitivity assay.
Radiosensitivity of all cell lines was tested with a 96-well plate clonogenic assay, developed by Grénman and colleagues (23, 24). The radiosensitivity of a cell line was defined as the area under the survival curve, with measurements of the survival fraction at 6 different doses, each repeated at least 3 times. When a comparison was made between radioresistant and radiosensitive cell lines, the cutoff was set at a median area under the curve of 2.0.

RNA collection after irradiation.
Cells were irradiated using a ^{137}Cs irradiation unit with a dose rate of 0.662 Gy/min. Mock-irradiated cells were harvested for all cell lines, as well as cells at 2 and 6 hours after 4 Gy. At the given time points, cells were rinsed with ice-cold PBS twice and then collected in RNA-Bee (Campro Scientific).

RNA isolation from cell lines

All steps from RNA isolation to microarray hybridization were performed at the Institute's central microarray facility. Cells in RNA-Bee were used to extract total RNA. The sample was then split into two for analysis of miR and mRNA separately. mRNAs were further purified using the RNeasy Mini Kit and the RNase-Free DNase Set from Qiagen. The RNA was isolated and DNAse treated using the spin columns according to the manufacturer's instructions. The Agilent 2100 Bioanalyzer was used to confirm the presence of intact RNA.

mRNA/miR microarrays in cell lines

mRNA.
Biotin-labeled cRNA was generated using the Illumina TotalPrep RNA Amplification Kit (AMIL1791, Ambion Inc.). Briefly, to synthesize biotin-labeled cRNA, 350 ng of total RNA was reversed transcribed and subsequently amplified and labeled with biotin (*in vitro* transcription). Next, the cRNA (1,500 ng per array) was hybridized to v3 Illumina bead arrays according to the manufacturer's instructions (Illumina, Inc.). Array signals were developed by Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences) following the BeadChip manual. Fluorescence intensities were measured with the scanner and averaged per probe. Background adjustment was done using the method from the affy package, after which data were $\log_2$-transformed and robust spline normalized. As a final step, annotations were updated using the lumiHumanAll package (25) in R and subsequently the data were

aggregated per gene symbol: data from probes with the same gene symbol and a correlation greater than 0.7 were averaged.

microRNAs.
Using the Exiqon miRCURY LNA microRNA Array kit (fifth generation), 1 µg total RNA was labeled with Hy3 and hybridized in a TECAN HS4800 Hybridization Station against the slides together with a reference pool of all samples (Hy5). The slides were scanned in a DNA Microarray Scanner (Model G250B, Serial number US22502518) from Agilent Technologies, which uses Scan Control software (Version A.6.11). After subtraction of the mean background signal, arrays were $\log_2$-transformed and normalized using the LOWESS method (using Imagene 6.0 software).

Patient series

Patient selection.
Thirty-four patients treated at The Netherlands Cancer Institute (Amsterdam, the Netherlands) between 2002 and 2010 were selected as a validation cohort. To avoid confounding by the addition of surgery or chemotherapy, a cohort consisting of patients with T2-3 laryngeal cancers was compiled. These patients were all treated with radiotherapy alone with a curative intent. The series was designed to be a matched cohort of 17 patients with local recurrences matched with 17 local cures. There were no significant differences between groups with and without local recurrence in age, gender, subsite, T-stage, or treatment year (Table 4.2).

Baseline characteristics			
		Cures	Recurrences
N		17	17
Sex	Male	59%	59%
	Female	41%	41%
Age (years)	Average	68	67
Treatment year	Average	2007	2007
T-stage	T2	65%	59%
	T3	35%	41%
Subsite	Glottic	47%	47%
	Supraglottic	53%	53%
Follow up (years)	Average	3.9	3.7

Table 4.2. Patient characteristics for the 34 patients in the validation cohort.

miR extraction.
Using the Roche High Pure miRNA Isolation Kit (REF: 05080576001), miRNAs were extracted from pretreatment biopsies. Briefly, 5 slides of 5-µm thickness were deparaffinized and macrodissected, assuring that the sample consisted of at least 50% tumor cells. miRs were further purified according to the manufacturer's instructions.

miRNA library preparation and sequencing

The total RNA samples were quality-controlled and quantified with the Agilent Technologies 2100 Bioanalyzer, using the RNA 6000 Nano kit. One microgram of total RNA in a volume of 5 µL was used as input for the miR library preparation for Illumina sequencing (SR 50bp) using the TruSeq Small RNA Sample Preparation Kit (RS-200-0012) and Guide (Part # 15004197 Rev. E). Shortly, stepwise RNA ligation of 3' and 5' adapters to miRs introduce a specific index to every sample. The product was PCR-amplified and pooled and purified using a 6% PAGE gel. Fragments of 145 to 160 bp were cut from the gel, washed and concentrated by ethanol precipitation, and resuspended in nuclease-free water. The small RNA library pools were quantified using a DNA 7500 chip with the Agilent Technologies 2100 Bioanalyzer. The pools were diluted to a concentration of 2 nmol/L and passed on for sequencing onto an Illumina HiSeq2000 machine and a stretch of 50 bp was sequenced according to manufacturer's instructions. The FAST-Q data from the run were analyzed and quantified by comparing the data to the miR databases.
Sequence reads (51 bp) were mapped using the mirExpress pipeline. The reads were trimmed for adapter sequences upon alignment. During the alignment, the identity was set to 0.9. Human mature and precursor sequences were downloaded from miRbase (version 20). The miR expression results that were generated for each sample were combined for further analysis. miR counts were normalized to 100,000 reads per patient.

Analysis

Time course analyses were performed using the Biometric Research Branch (BRB) ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html). This is a tool that performs a regression analysis of time course data, finding patterns that correlate with time, class, or both. Pathways and networks were analyzed through the use of Ingenuity Pathway Analysis (IPA; Ingenuity Systems, www.ingenuity.com). Cell survival curves were generated and analyzed in GraphPad Prism 6.0. All other analyses were performed in R (26), using the Bioconductor packages (27) and our own scripts.

miR target selection

Because most miR–mRNA interactions are predicted interactions on the basis of the complementarity of their RNA sequences and not on experimentally validated interactions, a collection of the most likely mRNA targets was generated for each miR by analysis of validated interaction data from external databases. A maximum of 750 mRNA targets per miR were selected on the basis of our own prediction model trained to predict experimentally validated targets from Tarbase 6.0 (28) on miR and target properties from TargetScanHuman 6.2 (14, 29). A list of these 146,898 interactions is available online.

Results

Data overview

All tested cell lines responded to irradiation by profound changes in gene expression. To investigate whether this response correlates with radioresistance, we determined the abundance of 18,913 unique mRNAs at 0, 2, and 6 hours after 4 Gy and of 279 unique miRs at 0 and 6 hours after 4 Gy in 32 HNSCC cell lines (Figure 4.1).

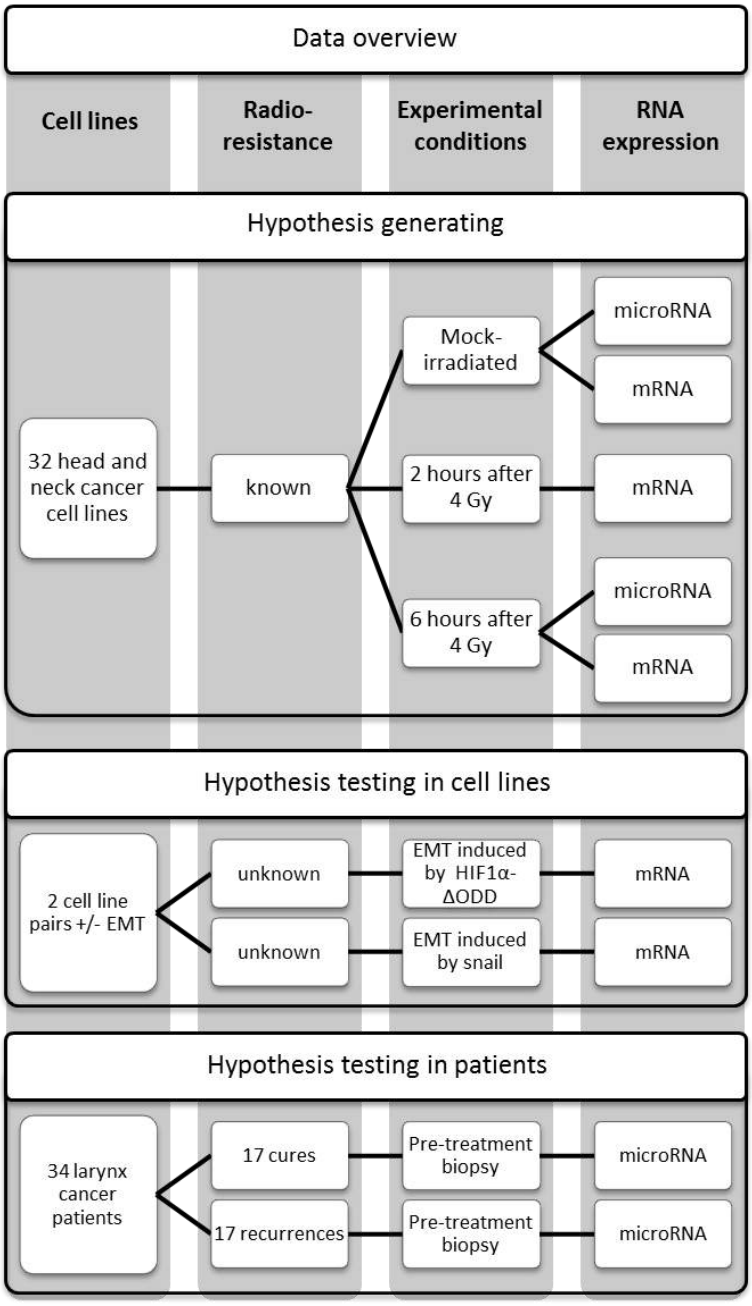

Figure 4.1. Overview of data.

MiR/mRNA expression changes 2 and 6 hours after 4 Gy do not correlate with radioresistance

Thousands of mRNAs and miRs showed expression changes in one or more of the cell lines in response to 4 Gy. The time course plug-in in BRB array tools identifies cell lines with similar gene up- or downregulation after irradiation. An expression response pattern common to all 32 cell lines involved 175 genes (Figure 4.2), none of them encoding miRs. When analyzing these common response genes in IPA, the most significant canonical pathways were associated with protein ubiquitination, cell-cycle regulation, and DNA double-strand break repair.

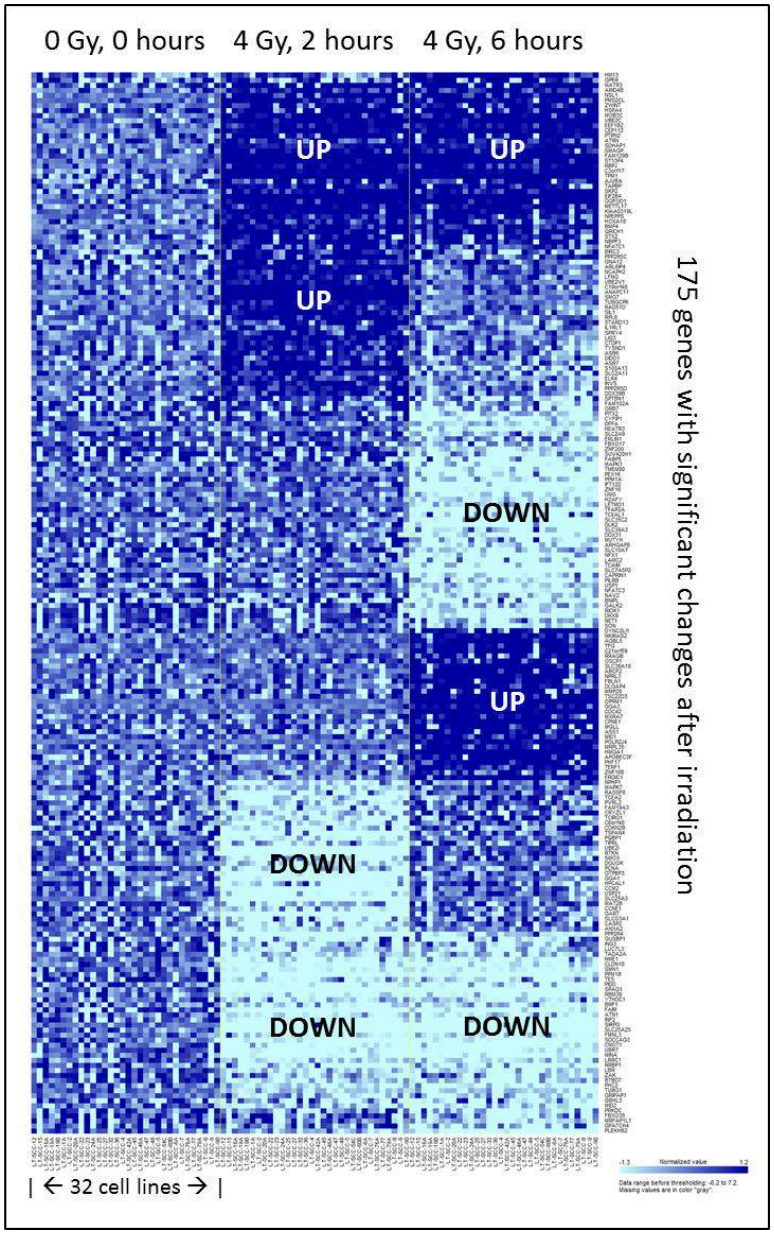

Figure 4.2. Heatmap of common response, 2 and 6 hours after irradiation: Heatmap of the common response to 4 Gy irradiation in all 32 cell lines, adapted from the BRB-array tools time course plug-in output.

When genes with an altered expression 6 hours after 4 Gy (compared with baseline expression) were subjected to cluster analysis, 2 main response clusters became evident. Genes that were different between the 2 response clusters were analyzed in IPA, which showed that 11 cell lines in the first cluster had an activated TP53 and HNF4A response, whereas this response was inhibited in the other 21 cell lines. However, the 2 clusters showed no correlation with radioresistance (*t* test; $P = 0.82$).

The time course plug-in also searches for response patterns that are significantly different between 2 groups. Here we found that changes 2 and 6 hours after 4 Gy did not differ between the 14 radiosensitive and 18 resistant cell lines, neither in mRNA nor in miR expression.

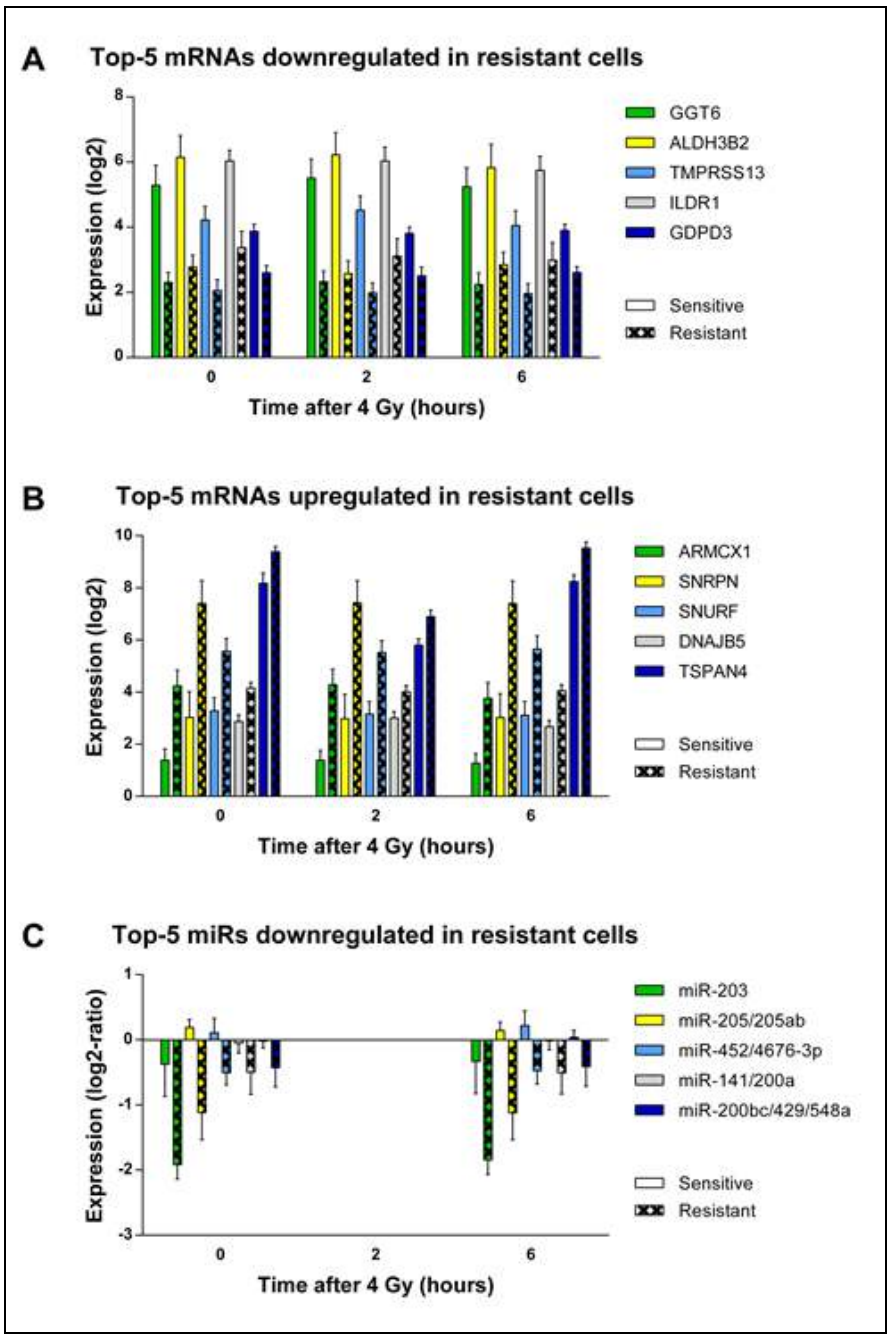

Figure 4.3. MiR and mRNA expression differences between resistant and sensitive cell lines. mRNA and miR expression differences between sensitive and resistant cell lines over time. The differences between sensitive and resistant cell lines at the individual time points were all individually significant (t test; P < 0.05), except for miR-141/200a and miR-200bc/429. The differences between sensitive and resistant cells for these two miR families were only significant, when the measurements at both time points were considered. Error bars, mean ± SEM.

mRNAs and radioresistance

The BRB time course plug-in further analyzes the difference between sensitive and resistant cell lines, independent of the time response. In this analysis, 1,226 genes with a stable expression over the 3 time points significantly correlated with radioresistance using a false discovery rate cutoff of <0.05 (Supplementary table 4.1). In addition, separate *t* tests were performed between the expression of the sensitive and resistant groups for each of the 3 time points. The 3 resulting *P* values were then pooled per gene. The expression over time for the top 5 positively and negatively correlated genes (i.e., with the lowest pooled *P* value) is shown in Figure 4.3A and B. An IPA showed that these 1,226 genes corresponded mostly with the following molecular and cellular functions: cellular movement, cellular development, cellular growth and proliferation, cell-to-cell signaling, and interaction and cell morphology. These functions are suggestive of a role for EMT, which describes a process in the cell that leads to loss of polarity, increased migratory and invasive capacity, and reduced cell–cell contact (30).

Identification of miRs that correlate with radioresistance

To find driving miRs that influence radioresistance, we set 3 separate requirements: (i) to select miRs that were actively degrading their mRNA targets, there had to be a negative correlation between miR expression and expression of its targets; (ii) a correlation between miR expression and radioresistance; and (iii) an inverse correlation of the target expression with radioresistance (compared with the miR–radioresistance correlation). Using these criteria, the chance of finding false-positive results is brought down to a minimum and only relevant miRs are identified.

For this analysis, miRs and mRNAs were filtered on the basis of the interquartile range (IQR) of expression between the 32 cell lines to exclude uninformative values. This left 200 miRs and 13,041 mRNAs with an IQR higher than 0.5 for the analysis. Of the 200 miRs, 39 were discarded because they had fewer than 5 predicted targets. After the filtering steps, the remaining 161 miRs had an average number of 506 predicted mRNA targets, as defined by our *in silico* generated miR–mRNA interaction database. Of these 161 miRs, 37 had a significantly negative miR target Pearson correlation after multiple testing correction. *P* values for the correlation between each miR and its targets were calculated using a two-sided *t* test of the Pearson correlations of the predicted mRNA targets for each miR versus the Pearson correlations of all other (random) mRNAs with the miR expression. *P* values for the correlation between mRNA targets and radioresistance were calculated using the same

approach, comparing the difference between all *P* values for the Pearson correlations between the targets and radioresistance versus all *P* values for the correlations between the nontarget mRNAs and radioresistance. *P* values for the difference in miR expression between sensitive and resistant cell lines over the two time points were obtained using the BRB time course plug-in. A significant correlation of the miR and its targets with radioresistance was observed for 12 of these 37 miRs, belonging to 10 different miR families (Table 4.3). Expression over time for the top 5 miR families can be seen in Figure 4.3C. Of interest is that 292 of the earlier identified 1,226 mRNAs that were significantly correlated with radioresistance are being regulated by one of these 12 miRs.

miRs correlated with radioresistance						
1. miR name	**2. No of predicted mRNA targets**	**3. Significant negative miR-mRNA targets correlation? (p-value)**	**4. MiR expression in resistant cells up or down?**	**5. Correlation with radioresistance**		**6. miR function**
				a. MiR only (p-value)	**b. All mRNA targets for this miR (p-value)**	
miR-203a	541	Yes ($1x10^{-5}$)	Down	$< 1x10^{-5}$	$3x10^{-10}$	Inhibit growth, self-renewal, migration, invasion and EMT
miR-205-5p	545	Yes ($3x10^{-26}$)	Down	$< 1x10^{-5}$	$8x10^{-9}$	Promote apoptosis, inhibit growth, migration, invasion and EMT
miR-452-5p	499	Yes (0.001)	Down	$7x10^{-4}$	$2x10^{-8}$	Reduce stem-like traits and tumorigenesis, EMT
miR-200b-3p$^{\$}$	562	Yes ($1x10^{-14}$)	Down	0.03	$1x10^{-15}$	Reduced proliferation, migration, invasion and EMT
miR-429$^{\$}$	562	Yes ($5x10^{-13}$)	Down	0.005	$1x10^{-15}$	Inhibit proliferation and EMT
miR-141-3p*	557	Yes ($1x10^{-5}$)	Down	0.02	$6x10^{-10}$	Inhibit EMT
miR-200a-3p*	554	Yes ($8x10^{-5}$)	Down	0.009	$1x10^{-9}$	Inhibit EMT
miR-7-5p	544	Yes ($3x10^{-14}$)	Down	0.04	$4x10^{-9}$	Inhibit invasion, self renewal and EMT, promote apoptosis
miR-138-5p	546	Yes (0.04)	Down	0.01	0.003	Inhibit proliferation, invasion, migration, modify DNA damage response
miR-34a-5p	539	Yes (0.0001)	Down	$2x10^{-4}$	$1x10^{-4}$	Inhibit proliferation, invasion, metastasis, stemness, EMT
miR-142-3p	522	Yes (0.03)	Down	0.04	$5x10^{-9}$	Maintenance of dendritic cells, inhibit growth and stemness
miR-33b-5p	483	Yes (0.0005)	Down	0.03	$2x10^{-5}$	Reduce proliferation, induce G1 arrest, cholesterol transport

Table 4.3. Relevant miRs correlated with radioresistance: Properties of the miRs and their associated mRNA targets that were significantly correlated with radioresistance. ***Column 1:*** *miR name.* ***Column 2:*** *The number of predicted mRNAs that are being targeted by this miR.* ***Column 3:*** *A significant negative correlation between the miR and its predicted targets indicates that this miR is actively degrading its targets.* ***Column 4:*** *The direction of the miR expression in the group of resistant cell lines.* ***Column 5a:*** *p-values from the BRB array tools time course plug-in, representing the correlation between radioresistance (AUC) and the expression of the miR over the 2 measured time points.* ***Column 5b:*** *p-value of a 2-sided T-test comparing the difference between all p-values for the Pearson correlations between the predicted mRNA targets and radioresistance versus all p-values for the correlations between the non-target mRNAs and radioresistance.* ***Column 6:*** *all references for the described miR functions can be found in Supplementary table 4.2.* $^{\$}$*Both member of miR family miR-200bc/429/548a.* **Both member of miR family miR-141/200a.*

EMT correlates with radioresistance

From the data described in mRNAs and radioresistance and Identification of miRs that correlate with radioresistance, it appears that the loss of miRs downregulating EMT mRNAs were significantly correlated with the intrinsic radioresistance of these 32 HNSCC cell lines.

To verify that EMT had a causal relation with radioresistance, we collected 2 HNSCC cell lines that had been forced to undergo EMT: UT-SCC-43A-Snail and FaDu-HIF1α(ΔODD). Both Snail and HIF1α are known transcription factors for EMT. In cell culture, the Snail- or HIF1α-expressing cells were clearly mesenchymal, whereas the respective control cells lines UT-SCC-43A and FaDu-cDNA3 had an epithelial growth pattern. In these pairs, we found that the cells that had undergone EMT were significantly more resistant to radiotherapy (Figure 4.4), with areas under the survival curve increasing from 2.7 to 3.9 ($P < 0.0001$) in the FaDu pair and from 2.6 to 4.6 ($P < 0.0001$) in the UT-SCC-43A pair.

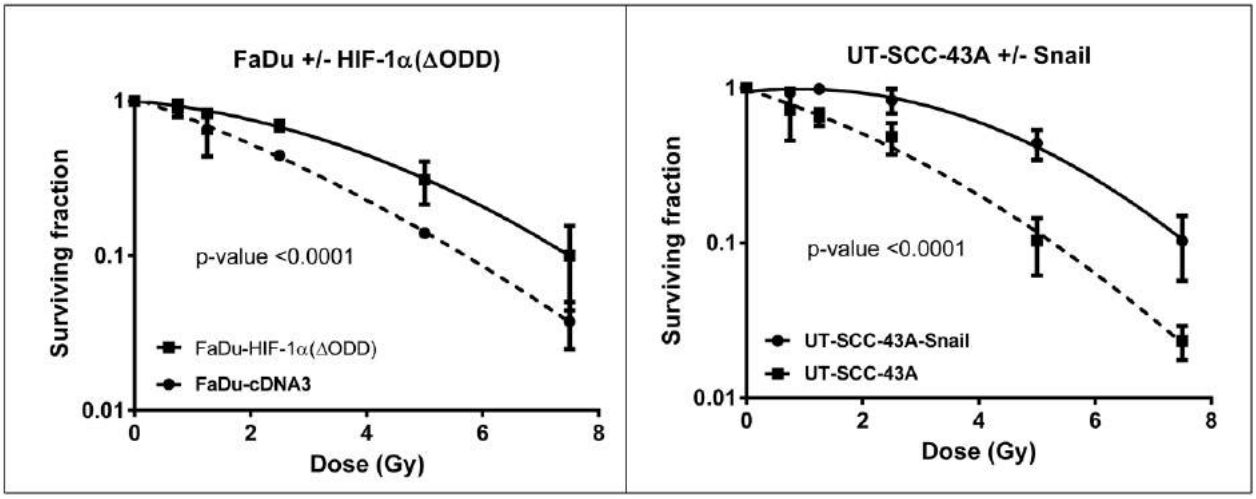

Figure 4.4. Induction of EMT causes radioresistance. Induction of EMT by HIF1α (left) or Snail (right) leads to increased radioresistance.

We further tested the correlation between radiosensitivity and processes known to influence radiotherapy response in the 32 cell lines, by using published gene sets for reactive oxygen species (31), hypoxia (32, 33), proliferation (34), stem cells (single marker CD44 and the set from ref. 35), p53 (constructed ourselves, Supplementary table 4.3), DNA repair (constructed ourselves, Supplementary table 4.3), and intrinsic radiosensitivity (8, 9). We also constructed our own HNSCC EMT signature from the two pairs of HNSCC cell lines in which EMT was induced. This signature was constructed from genes with a fold change greater than 2 or under 0.5 between parental and EMT-induced strains. In addition, only genes were selected that showed a fold change in the same direction (up- or downregulation) in both cell line pairs, which resulted in a set of 1,189 genes (Supplementary table 4.4).

For each cell line, a score was generated for each gene set, by either calculating the mean expression of the genes in the set or in the case of the HNSCC EMT signature by calculating

the Pearson correlation between the expression of the cell line and the average expression in FaDu-HIF1α(ΔODD) and UT-SCC-43A-Snail cell lines for these 1,189 genes. Next, scores for the gene sets were compared with the radiosensitivity values. Of the different gene sets, the HNSCC EMT gene set was the best predictor of radiosensitivity (linear regression *P*: 0.001) in the panel of 32 HNSCC cell lines, with a Spearman correlation of 0.74 ($P < 0.0001$). A plot of the HNSCC EMT score against the radiosensitivity is shown in Figure 4.5, the individual scores per cell line can be seen in Supplementary table 4.5.

Of note is that the two EMT-inducible cell lines, although HNSCC cells, were not part of the 32 cell line panel and thus were an independent test system, strengthening the interpretation of an EMT-based mechanism for radioresistance.

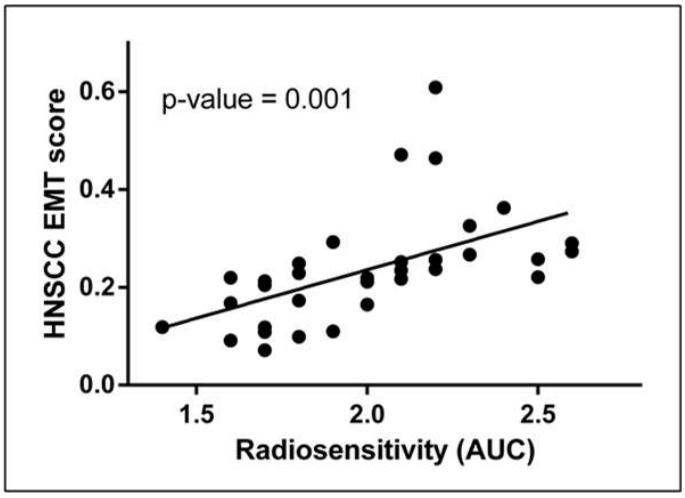

Figure 4.5. HNSCC EMT score versus radiosensitivity. Cells with a higher score for EMT (more mesenchymal) are more resistant to irradiation.

miRs predicting radiotherapy response in patients

The expression of the most significant miR in cell lines (miR-203) was tested in a pilot series of 34 patients with T2-3 larynx tumors treated with radiotherapy. The 12 top miRs were analyzed. When two groups created were divided by the median expression, a trend was seen for higher recurrence percentages with low expression of miR-452 (HR, 0.5; *P* = 0.1), miR-200b (HR, 0.7; *P*, 0.4), and miR-141 (HR, 0.6; *P* = 0.4). However, only low miR-203 expression was significantly correlated with local recurrence in a multivariate Cox regression (Figure 4.6; HR, 0.364; log-rank *P* = 0.04). These findings are in line with the cell line data, that is, loss of miR-203 expression leads to radioresistance.

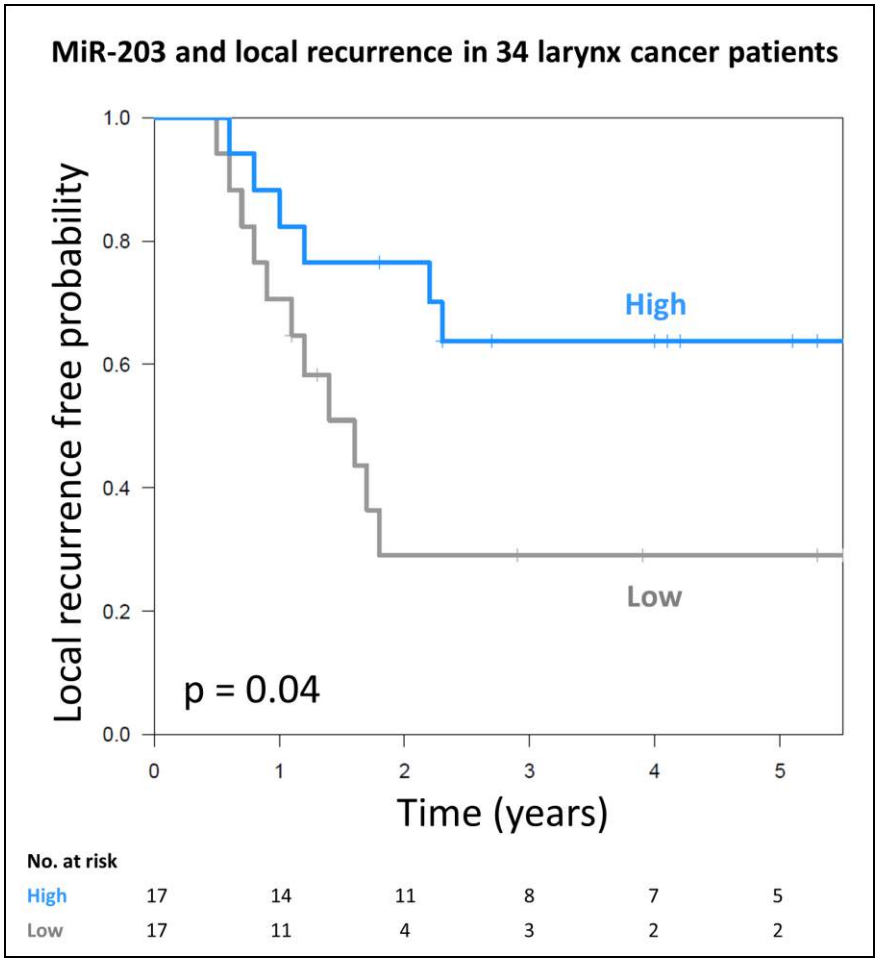

Figure 4.6. miR-203 in patients. Seventeen patients with a local recurrence after radiotherapy were matched with 17 patients without a local recurrence. Patients with a high miR-203 expression had a significantly higher cure rate. The mean survival in this curve is 50% because of matching 1:1.

 Discussion

It is not clear why some cells are radiosensitive and others are intrinsically radioresistant. By identifying the underlying mechanisms of radioresistance, it should become possible to personalize therapy where necessary, thereby achieving better treatment success rates. In this study, we correlated expression of miRNA and mRNA to intrinsic radiosensitivity of head and neck cancer. In our HNSCC cell line panel, we found that a low expression of certain miRs was strongly correlated with radioresistance. Different analysis methods led to the conclusion that EMT was an important factor in radioresistance, namely, the top correlating mRNAs, miRs, and gene sets were all involved in EMT and these findings were validated by testing two different cell lines engineered to undergo EMT, which caused an increase in resistance. Next, we have shown that low expression of the top miR (miR-203) predicting intrinsic radiosensitivity indeed corresponded to more local recurrences after radiotherapy in a patient series of laryngeal carcinomas. Because it has previously been reported that no major difference was detected in miR profiles among laryngeal, oropharyngeal, or hypopharyngeal cancers, we believe that this cohort could be representable for all of these subsites (36). It should be noted that results were obtained using multiple testing on a small series, needing further validation in a larger cohort of head and neck squamous cell carcinomas, preferably including head and neck tumors from different subsites.

Although separate EMT genes like fibronectin 1, Snail, Slug, and E-cadherin have already been associated with radioresistance (37–40), it has not been clarified why EMT would cause radioresistance. We hypothesize that simultaneous with acquiring a mesenchymal phenotype, the mechanisms by which cells can become more resistant to irradiation are altered. EMT is mainly a description of a phenotype, but the fact that the acquisition of this phenotype is correlated with radioresistance may indicate it affects at least one of the three known mechanisms that lead to resistance: less damage upon irradiation, better repair of irradiation damage, or less cell death upon damage.

A first hypothesis could be that the evasion of DNA damage could lead to radioresistance (31). In a recent overview, Watson proposed that mesenchymal cancer cells possess heightened amounts of antioxidants that reduce damage caused by irradiation-induced reactive oxygen species (ROS; ref. 41). Gammon and colleagues showed that within mesenchymal cancer cells under normoxic conditions, a subpopulation of cells with low oxygen and ROS levels can be found (42).

Second, a more effective DNA damage repair system can lead to increased survival of cells after radiotherapy. This appears to be the case in breast cancer cell lines, in which it was shown that HOXB9 induces both EMT and confers resistance to ionizing radiation by accelerating the DNA damage response (43). In another report, it was shown that ATM-mediated Snail serine 100 phosphorylation regulates cellular radiosensitivity (44).

Finally, damaged cells can evade cell death and thereby survive irradiation. Kurrey and colleagues propose a model in ovarian cancer, in which EMT transcription factors Snail and Slug can antagonize p53-mediated apoptosis (40). TGFβ is also known to simultaneously invoke EMT and block apoptosis via PI3K signaling (45). In addition, another EMT inducer, SIP1, has been ascribed antiapoptotic properties (46). With the acquisition of an EMT phenotype, cells have been shown to increase autophagy: a lysosomal degradation pathway that can be used to increase survival of cells (47). Rouschop and colleagues demonstrated that inhibition of autophagy sensitized xenografts to irradiation (48).

In an attempt to confirm these hypotheses, we tested different gene sets for reactive oxygen species, DNA repair, cell-cycle phase, and several means of cell death against the EMT gene set (Table 4.4). From these analyses, it appears that there is no single explanation for the radioresistance of the mesenchymal phenotype. The acquisition of a heightened EMT gene expression profile corresponds to a higher expression of genes known to be expressed in G_2, genes involved in DNA double-strand break repair and autophagy. This indicates that mesenchymal cells might become more resistant to radiotherapy by prolonging time spent in G_2, more efficient double-strand break repair, and the use of autophagy as a possible mechanism to evade cell death. ROS scavenger or apoptosis gene sets showed no correlation with expression of EMT genes.

Hypothesis for increase of intrinsic resistance	Gene set	Correlation with EMT gene set in 32 HNSCC cell lines	UT-scc-43A-snail (compared to control)	FaDu_HIF (compared to control)
1. Less damage	ROS-scavengers	-0.3	down	down
2. Better repair of damage				
2a. More time in checkpoint	G2 checkpoint genes	**0.5***	**up**	**up**
2b. Better DNA repair	NHEJ	**0.6***	down	up
	HR	**0.4***	**up**	**up**
	BER/SSBR	0.0	down	down
3. Less cell death	Apoptosis	-0.1	down	up
	Necrosis	-0.1	down	same
	Autophagy	**0.3***	**up**	**up**

*Table 4.4. Results of testing gene sets for reactive oxygen species, DNA repair, cell cycle phase and several means of cell death against the EMT gene set. Spearman's rank correlations. * p-value <0.05.*

Our study is the first to identify miRs with their mRNA targets that are involved in radioresistance in HNSCC. By analyzing miRs together with their targets, a more realistic representation of what occurs in cells can be obtained. A pitfall remains the allocation of the correct targets to every miR. Despite this possible confounding effect of wrongly allocated targets in the analysis, when studying the effect of all targets of one miR as a group, a reliable target effect can be observed. Future studies into correctly defining miR targets should improve this analysis method. The potential advantage of discovering miRs that are correlated with resistance is that, when used as therapeutic agents, they are able to target many genes at once, frequently within one pathway or network (49).

We observed that constitutive but not radiation-responsive genes correlated with radioresistance. These findings are consistent with findings of Birrell and colleagues on the yeast deletion mutant library (50) and the findings in the gene expression series of Amundson and colleagues who concluded that in the NCI-60 cell line panel "basal expression patterns discriminated well between radiosensitive and more resistant lines, possibly being more informative than radiation response signatures" (8).

In conclusion, the pre-irradiation miR-203 status, determined by integrative miR and mRNA analyses, was the most powerful predictor of radioresistance in our HNSCC cell line panel. This EMT-inhibiting miR was decreased in patients with a local recurrence after radiotherapy. The fact that radioresistance could be best predicted from baseline expression suggests that future studies into intrinsic resistance should not focus on response to irradiation. If these findings can be translated to the clinical setting, it should be possible to predict radiotherapy outcome from a pretreatment sample.

The next step would be to reverse EMT *in vivo*, possibly by restoring expression of miR-203. Because one miR can target many genes, EMT caused via different routes could potentially be inhibited by a single miR. Inhibition of EMT *in vivo* could not only make cells more radiosensitive but also more chemosensitive and less invasive, which together should lead to better patient survival.

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Supplementary information

Due to the size of the files, supplementary information for this chapter is only available online. Below are the hyperlinks to the corresponding supplementary tables:

Supplementary table 4.1 lists 1226 genes significantly correlated with radioresistance from a BRB time course plug-in analysis.

Supplementary table 4.2 describes the references used for the definition of miR functions stated in table 4.3.

Supplementary table 4.3 lists the genes in our p53 and DNA repair signatures.

Supplementary table 4.4 lists the 1189 genes in the HNSCC-EMT signature.

Supplementary table 4.5 lists the HNSCC EMT-scores for the 32 cell lines.

CHAPTER 5

Comparing hypoxia signatures in head and neck cancer

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Submitted for publication.

Abstract

BACKGROUND AND PURPOSE

Patients with hypoxic tumors poorly respond to radiotherapy and could benefit from hypoxia modification during radiotherapy. To identify these tumors, various gene expression profiles characteristic for hypoxic tumors have been suggested.

MATERIALS AND METHODS

Published profiles for hypoxia and *in vitro* obtained gene sets with early and late hypoxia response genes were compared using expression data from 224 head and neck cancer patients from three different datasets. The ability to predict local recurrence after chemoradiotherapy was tested for the different profiles.

RESULTS

Although only 3 genes were similar in the four validated hypoxia profiles, the profiles showed a near complete correlation with each other in categorizing the 224 patients. The published signatures correlated with the *in vitro* developed late hypoxia response, not with the early hypoxia response genes. Interestingly, the early hypoxia profile better predicted local recurrence after chemoradiotherapy.

CONCLUSIONS

Different sets of genes can be used interchangeably to study hypoxia status of tumors. Four published profiles were related to chronic rather than to acute *in vitro* hypoxia, while the acute profile better predicted local recurrence. For a better prediction of hypoxia status and the risk of recurrence, acute hypoxia profiles should be incorporated into existing models.

 Introduction

Head and neck cancer

The average overall survival for head and neck cancer patients is around 50% [1], but this varies greatly among different groups of patients. Applying clinical (TNM) staging to create different prognostic groups can only explain survival variation for 25% [2, 3, 4, 5]. We have previously shown that the addition of a prognostic gene expression profile can improve outcome prediction, suggesting that a substantial part of the survival variation is explained by tumor biology [6].

Hypoxia affects treatment outcome/prognosis

One of the most studied biological factors affecting prognosis of head and neck cancers is tumor hypoxia [7]. Tumor cells can become hypoxic by chronic (diffusion limited) and acute (perfusion limited) mechanisms, which can have different effects on tumor cells and their microenvironment. Which of the two has the most prognostic implications is still unclear [8]. Because oxygen is essential to cause DNA-damage upon irradiation, hypoxic cells respond poorly to radiotherapy [9, 10, 11, 12]. Since approximately two third of all head and neck cancer patients is (partly) treated with radiotherapy, hypoxia can be a great obstacle in the treatment of these tumors [13]. A meta-analysis of clinical trials showed that *in vivo* modification of the acute and/or chronic oxygen status during radiotherapy can improve survival of head and neck cancer patients, demonstrating that hypoxia is an important factor in radioresistance [14]. Unfortunately, the hypoxia modification therapy comes with added toxicity and the benefit from hypoxia modification was modest in the whole series [14]. This led to the hypothesis that only patients with hypoxic tumors profit from such a therapeutic intervention, which was shown to be correct in two recent studies [15,16].

Selection of hypoxic patients

Since selection of patients appears to be of importance, a robust approach to quantify hypoxia is essential. Different techniques have been applied to evaluate the level of hypoxia in a tumor and its impact on radiotherapy response [7], including an oxygen-sensitive needle probe inserted into the tumor [17, 18, 19, 20], exogenous immunohistochemical markers (e.g. pimonidazole [21]), endogenous markers (e.g. HIF1-alpha [22, 23, 24] or carbonic anhydrase IX [16, 22, 25, 26]) and imaging techniques like MRI [27] and PET [28]. None of these techniques is currently used in clinical practice.

Hoping to better reflect the intricate cellular response to hypoxia, there have been reports of panels of markers or gene expression sets that correlate hypoxia status with prognosis [15, 22, 29, 30, 31, 32, 33]. Several published signatures have been validated to be prognostic or even predictive in head and neck cancer [15, 29, 30, 31]. These signatures appear to have only a few genes in common, raising the question which signature performs best for the assessment of the level of hypoxia within a tumor. In none of these series a distinction was made between acute and chronic hypoxia.

With the intent to better select patients for hypoxia modification the NIMRAD study was recently initiated, aiming to ‘prospectively validate a gene signature that can be used in

clinical practice to personalize treatment and select appropriate patients for hypoxia modifying treatment' [34].

Study goals

We aimed to study the differences between the published hypoxia signatures that have been validated in head and neck cancer. First, we identified hypoxia signatures that have been validated to be prognostic or even predictive in head and neck cancer. We compared the genes included in these signatures and next the uniformity of these signatures in the classification of head and neck cancer patients into a 'hypoxic' and 'less hypoxic' group. In addition, we sought to compare these signatures with expression data of cell lines subjected to chronic/acute hypoxia. Lastly, the ability of the different signatures to predict radiotherapy response was tested in a series of 91 head and neck cancer patients who underwent chemoradiotherapy.

Materials and methods

Published hypoxia gene sets

To our knowledge, four gene expression sets for hypoxia that have been validated to predict outcome in head and neck cancer exist (table 5.1):

1. Winter et al. [29] profiled 59 head and neck cancer patients and obtained a hypoxia metagene signature, selecting genes whose *in vivo* upregulation coincided with the upregulation of 10 well-known hypoxia genes. The 99-gene signature correlated with recurrence free survival in a published series of 60 head and neck cancer patients mostly treated with surgery followed by radiotherapy [35].

2. *Buffa et al.* [30] used hypoxia-regulated genes to select co-expressed genes in three head and neck and five breast cancer studies. The resulting 51-gene signature was validated in 4 independent datasets.

3. *Toustrup et al.* [15] generated a 15-gene signature from *in vitro* experiments and an association of gene expression data from 58 head and neck cancer biopsies with various hypoxia levels from previous eppendorf hypoxia measurements. The 15-gene hypoxia classifier was validated and proven to be predictive for hypoxia modification (nimorazole) benefit in 323 patients treated in a randomized study of nimorazole versus placebo during radiotherapy for head and neck cancer.

4. *Eustace et al.* [31] generated a 26-gene reduced signature using the methods and starting genes from Buffa et al.. This signature was tested on 157 laryngeal cancer patients treated with radiotherapy alone or with carbogen and nicotinamide. The 26-gene signature predicted recurrence rate improvement upon hypoxia-modifying treatment.

Year	Authors	Genes	Method
2007	Winter et al.	99	Upregulation with well-known hypoxia regulated genes
2010	Buffa et al.	51	Upregulation with well-known hypoxia regulated genes in 3 HNSCC and 5 breast cancer series
2011	Toustrup et al.	15	In vitro experiments + correlation with eppendorf hypoxia measurements
2013	Eustace et al.	26	Reduced variant of Buffa et al., validated in ARCON series

Table 5.1: Overview of published hypoxia gene sets that have been validated in head and neck cancer.

In vitro hypoxia response data

To compare the published hypoxia signatures with acute and chronic hypoxia expression profiles, temporal transcription changes in response to hypoxia generated by Chi et al. were used [36]. They have studied hypoxia response patterns in epithelial cells using DNA microarrays. Gene signatures were extracted from cells at different time points between 1 and 24 hours under <0.02% or 2% oxygen [37]. Time points between 0-6 hours were used to describe early response and time points 12 and 24 hours late response, resulting in 4 signatures: early-0%, early-2%, late-0% and late-2%, consisting of respectively 70, 34, 65 and 29 unique gene symbols. These signatures were used for comparison with the four published signatures.

Patient data

To compare the classification of the different signatures, we used pre-treatment gene expression data of three different patient cohorts, comprising a total of 224 patients (table 5.2). More extensive patient characteristics for the cohorts can be viewed in the original publications and in Supplementary table 5.1-5.3.

Series	Patients	Site	Treatment	Material	Assay
Stage III-IV HNSCCs	91	All head and neck	Radio-chemotherapy	Fresh frozen	Dual channel Operon microarray
Larynx / oropharynx	99	Larynx	Radiotherapy	Fresh frozen	Illumina beads microarray
T2-3 larynx	34	Larynx	Radiotherapy	Paraffin	RNAseq

Table 5.2. Summary of characteristics of the three patient series.

The first series of 91 patients treated with radiochemotherapy was previously published by Pramana et al [38]. Gene expression profiles were obtained from fresh-frozen pre-treatment material, analyzed using dual-channel Operon microarray slides. Follow-up data were updated and annotations of reporters for different probes on the microarrays were updated to the latest HUGO gene symbols.
Data of the second series were partly published, methods are as described in de Jong et al.. For this analysis more patients were added to the series [39]. Briefly, gene expression of 99 fresh-frozen larynx and oropharynx carcinomas, all treated with single modality radiotherapy, was measured using the Illumina beads microarray platform. Annotations of

reporters for different probes on the microarrays were updated to the latest HUGO gene symbols.
The patient characteristics of the third series have been published previously [40]. This series consists of 34 larynx carcinomas, of which messenger RNA was isolated from paraffin embedded material and sequenced using the Illumina HiSeq2000, full methods for the mRNA extraction and sequencing can be read in the supplementary methods.

Testing signatures

All signatures consisted exclusively of genes that were upregulated under hypoxia. Therefore, the mean expression of the genes in each signature was calculated as a measure of hypoxia status for every tumor. In order to compare three patient series with expression data that were generated using different gene expression assays, scores were rank-normalized per signature between 0 and 1 for each of the three patient series before they were combined.

Hypoxia profiles and radiotherapy response

In order to study the effect of the different hypoxia signatures on (chemo-)radiotherapy response prediction, local recurrence rates for different hypoxia scores were compared in the chemoradiotherapy cohort. Per gene profile (or per group of corresponding gene profiles), patients were divided into two groups by the median rank. Kaplan-Meier statistics were used to assess the difference in recurrence free survival between two groups.

Results

Few overlapping genes in four different hypoxia gene sets

The four published gene sets for hypoxia that have been validated to predict outcome in head and neck cancer consisted of a total of 147 unique gene symbols. Of these gene symbols, 82% was only present in one of the four signatures, whereas 2% of the genes was present in all four signatures: *ALDOA*, *P4HA1* and *SLC2A1* (figure 5.1). Aldolase A is a glycolytic enzyme, the *P4HA1* gene encodes a component of a key enzyme in collagen synthesis and the *SLC2A1* (a.k.a. *GLUT-1*) gene encodes a glucose transporter.

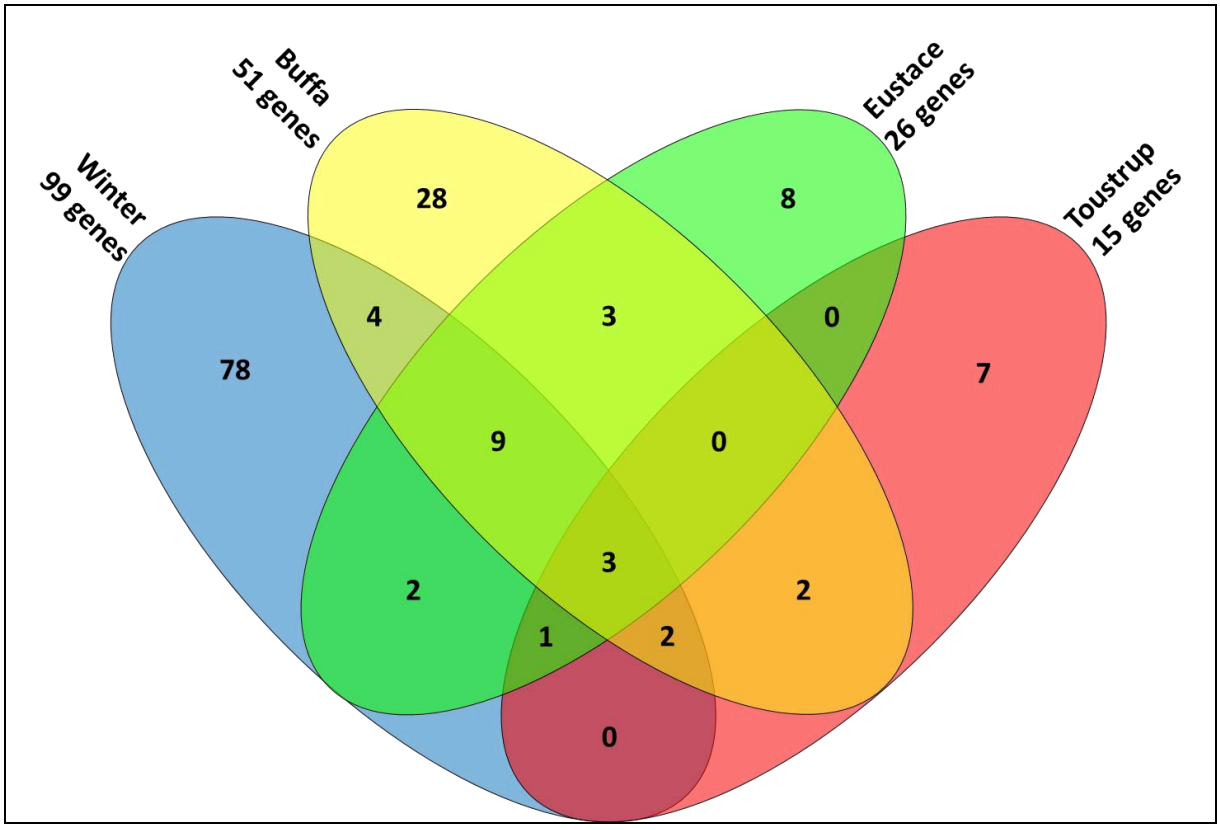

Figure 5.1. Four-way Venn diagram showing the overlapping genes in the 4 different signatures.

Classification of patients using four different hypoxia gene sets is nearly identical

Every tumor was ranked between 0 and 1 for each signature, representing the average expression of the genes in the different signatures. Scores between different signatures could then be compared, based on their classification of the 224 patients. As can be observed in figure 5.2, the average Spearman correlation between scores assigned by the different signatures was highly significant, with an average correlation of 0.82 (range 0.71-0.90, all p-values < 0.0001). This indicates that the four signatures rank patients in an almost identical manner.

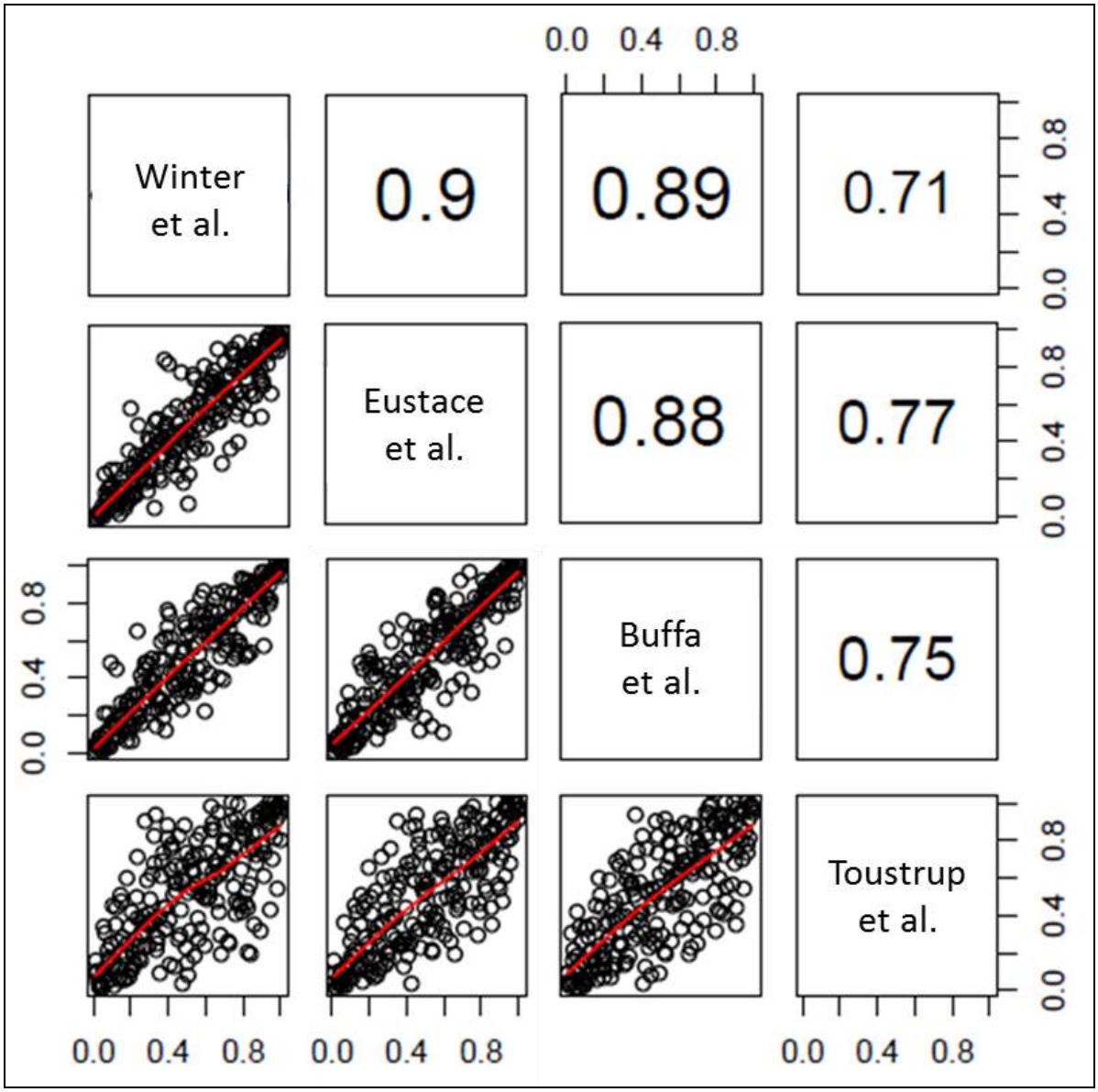

Figure 5.2. Spearman correlations (upper right panels) and scatterplots (lower left panels) of all possible pairs of hypoxia signatures for 224 patients. All Spearman correlations were significant at the p < 0.0001 level.

Published hypoxia gene sets resemble *in vitro* chronic hypoxia response

Scores for the four published gene sets and four *in vitro* hypoxia gene sets (early and late response to 0% and 2% oxygen) were generated for all 224 patients. The average Spearman correlations between the scores for the published profiles and late-0% or late-2% O_2 response profiles were 0.60 and 0.49 respectively (both p<0.0001). The average correlations with early response were -0.09 (p=0.2) and 0.23 (p<0.001) for early-0% and early-2% O_2 respectively. All correlations and the corresponding scatterplots can be seen in figure 5.3.

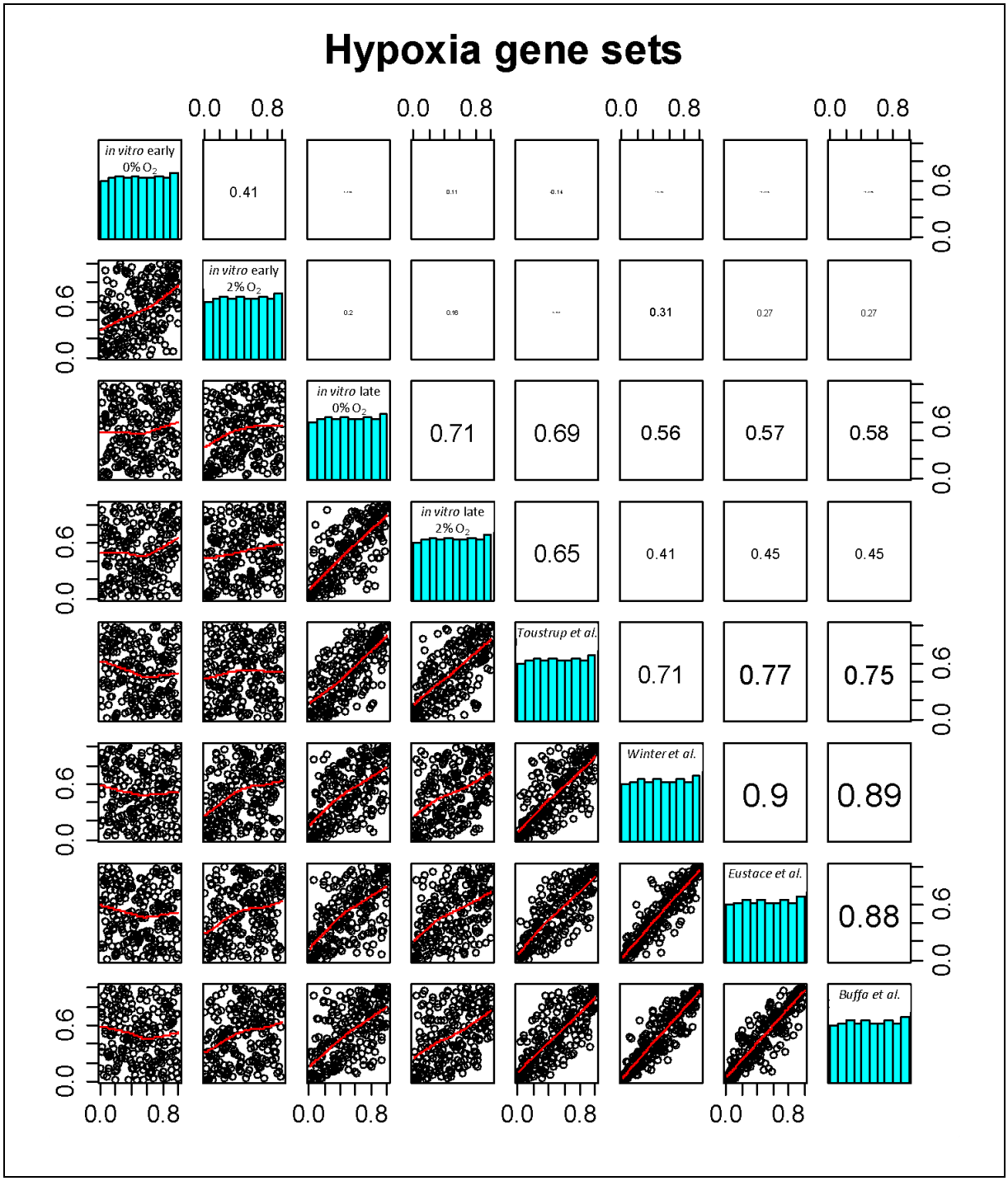

Figure 5.3. Correlation of all published and in vitro hypoxia profiles: Spearman correlations (upper right panels), histograms of the normalized (0-1) scores for the signatures (diagonal panels) and scatterplots (lower left panels) of all possible pairs of hypoxia signatures for 224 patients. The printed size of the Spearman correlations is a representation of the actual absolute size of the correlation.

A clustering of the scores for the 224 patients can be seen in figure 5.4. The dendogram to the left of the heatmap shows that, again, the four published gene sets clustered together. Interestingly, the two *in vitro* profiles of late response to hypoxia clustered with these

published profiles (cluster 1), whereas no correlation was observed with the *in vitro* profiles of early response to hypoxia (cluster 2).

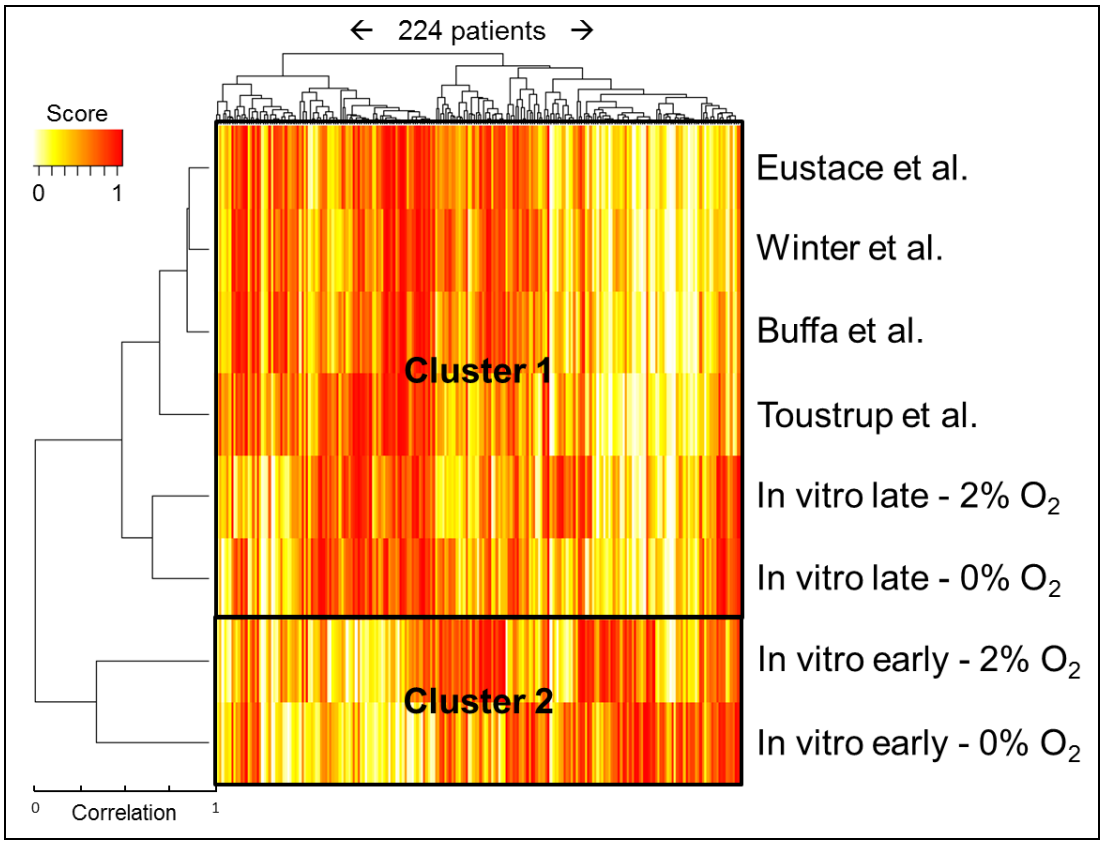

Figure 5.4. Heatmap showing the scores for the expression of different genes/gene sets in 224 patients.

In vitro early hypoxia response profile predicts recurrence in 91 chemoradiotherapy patients

The predictive value of the different hypoxia profiles on local recurrence rate after therapy was tested on the 91 chemoradiotherapy patients. Per gene signature, patients were divided into two groups by the median rank. Kaplan-Meier statistics were used to assess the difference in recurrence free survival between the two groups. Of the four published and four *in vitro* gene sets, only the '*in vitro* early 0% O_2' set showed a significant difference (log rank p-value = 0.02) between high and low expression: patients with a low expression of *in vitro* early hypoxia genes had a lower recurrence percentage with a hazard ratio of 3.1 (95%CI: 1.1–8.6). Curves and hazard ratio's for all signatures can be seen in Supplementary figure 5.1. Since scores for the 4 published profiles and the two late *in vitro* profiles were similar, they were averaged per patient to obtain a joint chronic hypoxia score. These average scores indicated that low expression of chronic hypoxia genes tends to give a better recurrence free survival (HR=1.8, 95%CI: 0.69-4.5, p=0.2, Kaplan-Meier curves in Supplementary figure 5.2). In this analysis the effect was not significant. To learn whether the effects of the acute and joint chronic hypoxia signatures were independent, a crosstab was made showing local recurrence percentages for high and low acute and chronic hypoxia (figure 5.5). High and low expressors were defined as above or below the median expression for the whole group.

Next, a Kaplan-Meier curve was made for three groups: low acute and chronic hypoxia, high acute or high chronic hypoxia and high acute and chronic hypoxia (figure 5.5). The curves and the crosstab in figure 5.5 show that when both acute and chronic hypoxia expression scores were low, the chance of tumor recurrence was far lower than when both were high.

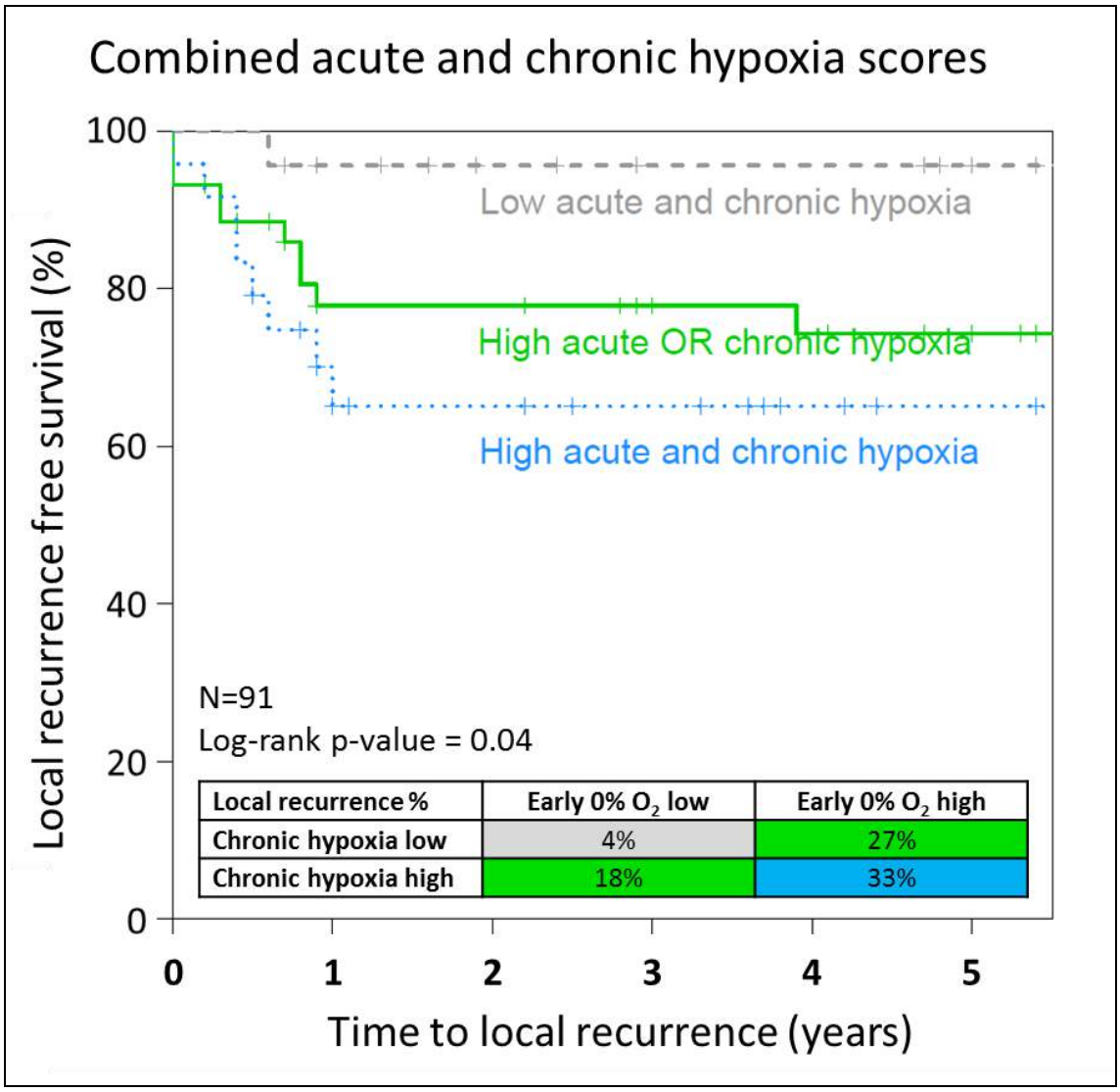

Figure 5.5. Kaplan-Meier curve of freedom from local recurrence for 3 groups: low acute and chronic hypoxia, high acute or high chronic hypoxia and high acute and chronic hypoxia. Crosstab of local recurrence percentage per subgroup in 91 chemoradiotherapy patients. Definition of chronic hypoxia: the average of scores for late 0% and 2% hypoxia, Toustrup et al, Winter et al, Eustace et al and Buffa et al gene sets. Samples were divided into two groups using the median. Cells are colored in a color corresponding with the line color in the Kaplan-Meier curve.

Discussion

We found that four published gene sets for hypoxia that have been validated to predict outcome in head and neck cancer had little overlap in terms of included genes. Nevertheless, they classified patients in an almost identical manner, indicating that they all reflect the same underlying process. This underlying biological process correlated with chronic, and not acute, *in vitro* hypoxia. While the validated prognostic profiles showed no resemblance to *in vitro* early hypoxia response, this acute response (and not the chronic response profile) was a significant predictor of local recurrence in 91 HNSCC patients treated with chemoradiotherapy.

Same classification, different gene sets

The phenomenon that signatures consisting of different genes can describe the same process, has been reported by Roepman et al [41]. They showed that multiple robust signatures to predict the presence of lymph nodes in head and neck cancer could be created from a larger group of predictive genes, which were not all needed to form an accurate predictor. Given the fact that over 4,000 genes are hypoxia-regulated, it seems reasonable to assume that multiple robust, but entirely different, hypoxia signatures can be assembled [42].

Acute and chronic hypoxia

The terms acute and chronic hypoxia are obviously simplified terms to describe a spectrum of hypoxic cells in a tumor [43]. While an absolute distinction between the two cannot be made, many suggestions for the separate origin, measurement and treatment of the two entities have been published [44, 45, 46, 47].

Janssen et al. employed various staining protocols to study acute and chronic hypoxia in head and neck tumors [45]. They showed that tumors contained on average 15% acute hypoxic (proliferating cells around temporarily non-perfused vessels) and around 30% chronic hypoxic areas (cells at a large distance from blood vessels). The two types of areas showed no overlap. This was also reflected in gene expression profiles of cells. Cells that had been under hypoxia for a short time, showed a very different gene expression as compared to cells that were hypoxic for longer periods of time [37]. As described by Lendahl et al. in a colon carcinoma cell line, 4,047 genes were hypoxia-regulated, of which only 52 were specific for acute (1 or 2 hour) hypoxia response, 144 genes were up- or downregulated by both acute and chronic (24 hour) hypoxia, whereas the majority of the genes (4,005) were chronic hypoxia specific [42].

Nonetheless, all creators of hypoxia signatures have tried to generate one signature for ‘general hypoxia’. The fact that these signatures correlated with *in vitro* chronic hypoxia could simply be due to the large excess of genes regulated by chronic hypoxia [42], but also to the methods used for the generation of the signature. For the Toustrup et al. profile an explanation could be that they correlated genes with eppendorf probe measurements. If indeed on average twice the amount of chronic hypoxic areas is present, as reported by Janssen et al, this could lead to a stronger correlation with chronic hypoxia genes. Winter,

Buffa and Eustace et al. started with 10 hypoxia 'seed genes' to develop their signatures. In our data, these 10 genes were not correlated with *in vitro* acute hypoxia and most showed some correlation to late *in vitro* hypoxia (Table 5.3).

10 seed genes											
Correlation with in vitro:	**ADM**	**SLC2A1**	**PDK1**	**ENO1**	**HK2**	**PFKFB3**	**AK3**	**CCNG2**	**CA9**	**VEGF**	**Average correlation**
early-0%,	-0.22	-0.17	-0.05	0.03	-0.18	-0.15	0.05	0.13	-0.05	-0.04	-0.06
early-2%	-0.05	0.11	-0.07	0.29	-0.09	-0.04	-0.12	0.02	0.18	0.09	0.03
late-0%	0.48	0.45	0.23	0.48	0.36	0.17	-0.27	0.06	0.24	0.32	0.25
late-2%	0.33	0.32	0.23	0.33	0.35	0.18	-0.12	0.14	0.27	0.24	0.23

Table 5.3. Correlation of 10 'seed genes' with in vitro acute and chronic hypoxia profiles.

Acute hypoxia and prognosis

The importance of acute hypoxia has been recognized for decades [48]. For example, Chan et al showed that a human lung squamous cell carcinoma cell line (H1299) became more radioresistant under acute hypoxia than under chronic hypoxia, with respective oxygen enhancement ratios of 1.96 and 1.37 [49]. Unfortunately, conclusive data on the separate and combined prognostic effects of acute and chronic hypoxia in head and neck tumors are lacking. This might be due to the fact that it is difficult to measure both types of hypoxia with immunohistochemistry.

Cutoff and effect size of hypoxia status

Using the median expression as a cutoff to create two groups, we found that patients with high acute or chronic hypoxia expression, had a 3.1 or 1.8 times higher risk of local recurrence, respectively. Although the latter was not significant, possibly due to the number of patients, the effect size appears comparable to previously reported hazard ratios for chronic hypoxia. Toustrup et al. found that the risk of locoregional recurrence was 1.85 times higher for "more hypoxic" tumors compared to "less hypoxic" tumors. Eustace et al. reported in their series of larynx carcinoma patients that the "more hypoxic" tumors receiving accelerated radiotherapy had a 5-year recurrence rate of 19%, while the patients with "less hypoxic" tumors had a recurrence rate of 9%. Winter et al. also reported recurrence-free survival, but compared the highest quartile to the rest of the patients. Using this method, the HR was 3.6 in a univariate analysis and 2 in a multivariate model. Buffa et al. reported a HR of 6.25, though the confidence interval (0.83-47.2) indicated a high level of uncertainty.

Hence for chronic hypoxia gene expression signatures, the general deduction is that more hypoxic tumors are approximately twice as likely to recur than the less hypoxic tumors. This effect could be underestimated due to a division of two hypoxia groups according to the median. Furthermore, acute hypoxia has not been studied in these series, but might well be more predictive than chronic hypoxia.

Conclusion

Different sets of genes can be used interchangeably to study the extent of hypoxia-driven gene expression in head and neck cancer. Although they scarcely contain overlapping genes, published gene sets for hypoxia that have been proven to be prognostic in head and neck cancer classify patients into the same riskgroups. These published sets all correlate with chronic and not with acute *in vitro* hypoxia-induced gene expression profiles. However, the acute hypoxia profile correlates better with the risk of recurrence after chemoradiotherapy in our series. Acute hypoxia gene expression should therefore be incorporated into existing hypoxia-based prediction models.

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Supplementary information

Due to the size of the files, supplementary information for this chapter is only available online. Below are the hyperlinks to the corresponding supplementary data:

Supplementary methods

Supplementary table 5.1: Patient characteristics series 1: 91 HNSCC stage III-IV radiochemotherapy patients.

Supplementary table 5.2: Patient characteristics series 2: 99 larynx/ oropharynx radiotherapy patients.

Supplementary table 5.3: Patient characteristics series 3: 34 larynx radiotherapy patients.

Supplementary figure 5.1 Kaplan-Meier curves for all published and *in vitro* hypoxia profiles.

Supplementary figure 5.2: Kaplan-Meier curve for the combined chronic hypoxia profile.

CHAPTER 6

General discussion

Contents

 6.1: The road to discovery of clinically relevant biomarkers for radiotherapy response

The research presented in this thesis describes studies into the individual biological tumor properties of head and neck cancer, using messenger- and microRNA data to predict which tumors will be more radioresistant and to gain insight into the mechanisms behind this. Eventually this should lead to a better understanding of the causes for radiotherapy failure allowing an up-front adaptation of therapy to give each individual patient the best chance of survival with the least amount of toxicity (1, 2, 3).

Since 2002, after the publications of Van de Vijver en Van 't Veer et al. showing that pre-treatment gene expression can be used for the successful prediction of survival in breast cancer patients (4, 5), there has been a huge influx of papers trying to replicate these results for different tumor sites. Various authors reported the discovery of a gene expression profile to predict outcome in head and neck cancer (6, 7, 8, 9, 10, 11, 12). Most series were small and very heterogeneous in terms of patient characteristics and treatment regimens used. Often gene expression profiles were not validated on independent series, which is particularly important when prognostic genes are selected from a set of almost 20,000 genes, even when the correct statistical methods are applied. Additionally, the reported prognostic gene expression profiles were not tested in a model with clinical factors that were already known to be prognostic. In the worst case scenario, one of these gene expression profiles would be a very complicated method to tell the gender of a patient (as mentioned previously being male is prognostically unfavorable) and not at all useful.

Keeping this in mind, we first questioned whether gene expression would be able to add prognostic power to known clinical factors in head and neck cancer. In **chapter 2**, we show that gene expression (HPV-status and a profile published by Chung et al.) can improve the prediction model and adds valuable information to known clinical factors. However, this series was heterogeneous (different subsites, HPV positive and negative tumors) and chemotherapy was administered concomitantly with radiotherapy.

In order to find a true predictor of response to radiotherapy, the next step was to study a more homogeneous series of patients, preferably all treated with only radiotherapy. Since gene expression could at the time of sample collection only be done on fresh frozen material, these scarce samples were recruited from various Dutch hospitals to collect a matched series of small larynx cancers, described in **chapter 3**. With the analysis of this small, but homogeneous series, we preferred a hypothesis-driven approach (test gene sets for known biological processes), as opposed to a data-driven approach (test all ~20,000 genes) for two reasons. Firstly, this reduces the number of tests: 10 gene sets versus ~20,000 separate genes, making the statistics more robust. To illustrate this: using a p-value of 0.05 (which is of course not advised for the analysis of 20,000 genes) the chance of finding a false

positive is 5%, meaning less than 1 out of 10 gene sets, but 1,000 false positives out of 20,000 tested genes would be found. Secondly, the hypothesis-driven approach will give results that are directly correlated to biological processes that could possibly be targeted to improve therapy. In this series we found cancer stem cell marker CD44 to be the only predictor of response to radiotherapy, which was validated on an independent series using immunohistochemistry (protein level). Since then many other authors have published this same finding, also in larger and non-laryngeal head and neck cancers (13, 14, 15, 16, 17, 18, 19).

A problem with the use of the hypothesis-driven approach is the acquisition of useful gene sets that correctly portray important biological processes. In neither of our patient series intrinsic radiosensitivity came up as a significant factor, while we know from clinical data that radiosensitivity measured by colony assays correlates with outcome after radiotherapy (20). We therefore concluded that we were not using an accurate messenger RNA set as a representative of this process and resolved to generate such a set. Another possibility was that messenger RNA levels alone were giving an incomplete picture of the active processes in the cell, since more factors can influence translation to protein. Among these are microRNAs, small pieces of RNA that can single handedly inhibit the translation of many messenger RNAs. The fact that it was reported that microRNA profiles were more accurate than messenger RNA profiles in the classification of poorly differentiated tumors (21), led us to hypothesize that they might also be more accurate in the prediction of intrinsic radiosensitivity.

Chapter 4 describes the discovery of a microRNA (miR-203), which downregulation strongly correlates with intrinsic radiosensitivity in cell lines and response to radiotherapy in a series of laryngeal cancer patients. The loss of miR-203 correlates with a biological process called epithelial to mesenchymal transition (EMT). The induction of EMT in cell lines is shown to decrease radiosensitivity.

Although a link between EMT and cancer stem cell marker CD44 has been described (22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34), we observed no correlation between CD44 expression and intrinsic radiosensitivity (**chapter 3**), nor a correlation between CD44 and miR-203 in 34 laryngeal cancer patients (unpublished results **chapter 4**, after acquisition of messenger RNA data for the same patients). This suggests that although there might be a link between EMT and cancer stem cells, not all cancer stem cells possess the same radiosensitivity and therefore both factors are independently important in the prediction of response to radiotherapy.

 6.2 Is more research needed?

After having studied 3 different patient series, we cannot conclude that HPV-status, Chung expression profile, CD44, hypoxia and miR-203 measurement on a pre-treatment biopsy should be the only markers we need to study in the future. Although important steps towards our understanding of head and neck cancer radioresistance, there are several reasons outlined below why more research is needed.

6.2.1 Patient numbers

First of all, the patient cohorts we have studied were all rather small, meaning just the largest effects in these series were statistically significant. For example, if we were to show a statistically significant (p-value < 0.05) effect for low versus high CD44 (or any other factor), assuming two groups of equal sizes, with an 80% probability to detect a statistically significant difference (power) in a group of head and neck cancer patients with a median survival of 2 years, a group 105 patients (65 events) would be needed to show the recurrence rate was twice as high (hazard ratio of 2.0), but only 17 patients (12 events) would be needed to show a five times worse recurrence rate (hazard ratio of 5.0) (35). To get an insight into patient numbers needed, different parameters can be entered into sample size calculators, for example on this website: sample size calculator. Keeping in mind that fairly large numbers of patients are needed to show a significant effect with a moderate difference between two groups, we could for example re-evaluate results found in **chapter 2**. Low CD44 expression appeared to be a favorable factor in the group of patients in **chapter 2**, but did not reach statistical significance. When compared to a similar but larger series recently published by Linge et al. (36), CD44 is significantly correlated with locoregional control as can be seen in figure 6.1. Meaning other factors in our analyses could have wrongly been judged to be 'insignificant', while they were only lacking sufficient patient numbers.

6.2.2 Cutoff values

Sample size will not only limit the detection to only the largest effects in a series, but also make it more difficult to find statistical significance if a factor is present in only a small subset of patients. Additionally, in many of our analyses, we split patients in two groups (using the median expression) for lack of knowledge of the actual cutoff. This is statistically sound to do if the cutoff is unknown and will produce stable results, but it might also miss factors that turn out to be important. In the unpublished plots in figure 6.2, it can be observed that if a cutoff at the first tertile instead of the median had been chosen, results would have been significant.

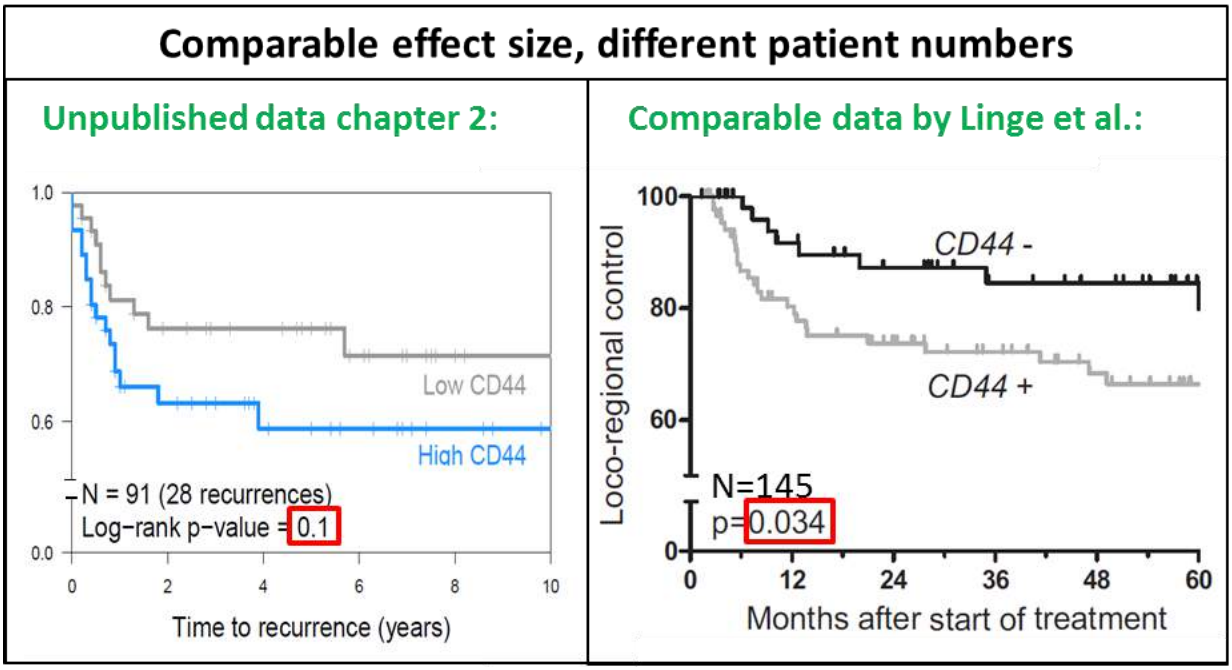

Figure 6.1: Kaplan Meier curves for CD44 in two groups in two different series. Left panel: unpublished plot from 91 patients in chapter 2, right panel: Curves for 145 patients, adapted figure 2D from Linge et al. (36).

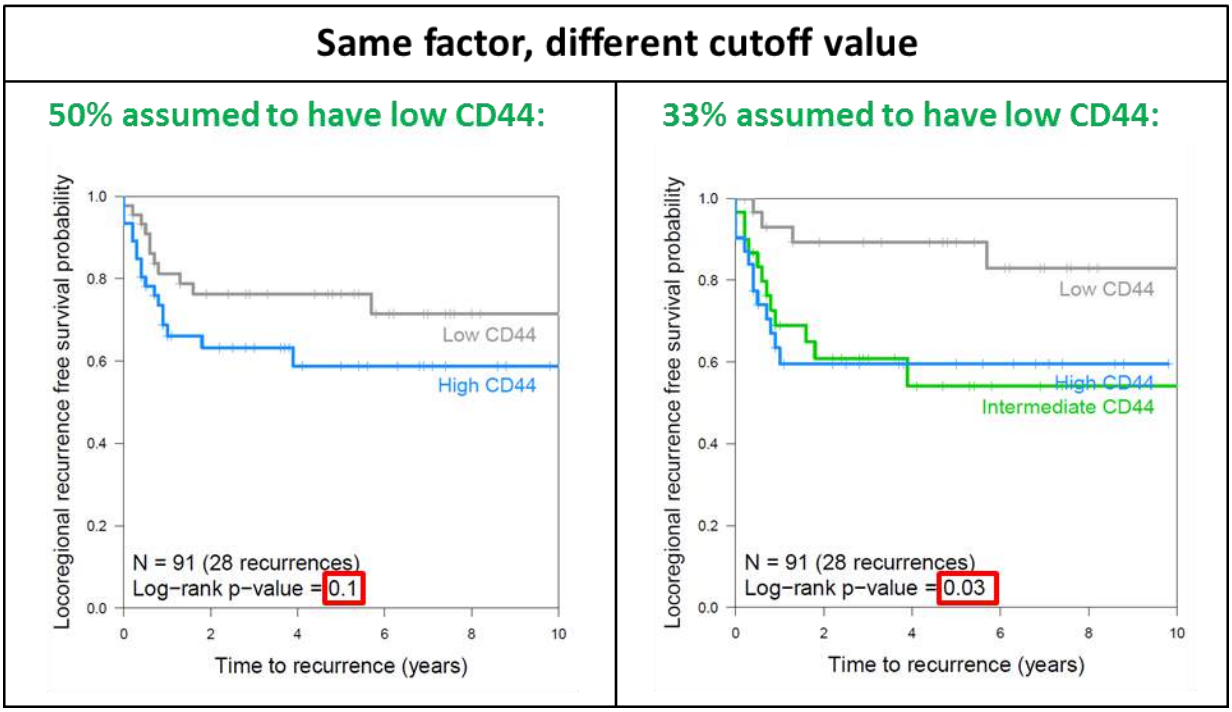

Figure 6.2: Kaplan Meier curves for CD44 in two or three groups. Unpublished plots from 91 patients in chapter 2. Comparison of a cutoff at the median (left panel) or in tertiles (right panel).

6.2.3 Interactions between factors

With small patient groups and numerous potential predictors of outcome, it is increasingly difficult to perform subgroup analyses or study different interactions between factors. Again, re-analyzing the data from **chapter 2**, where CD44 was not a significant factor, an interaction between HPV status and CD44 expression could have caused the 'insignificance' of CD44 in the original analysis (figure 6.3). Because laryngeal tumors are rarely HPV positive, this was not a confounder in the analyses of **chapter 3**, and again emphasizes the importance of the study of homogeneous groups of patients.

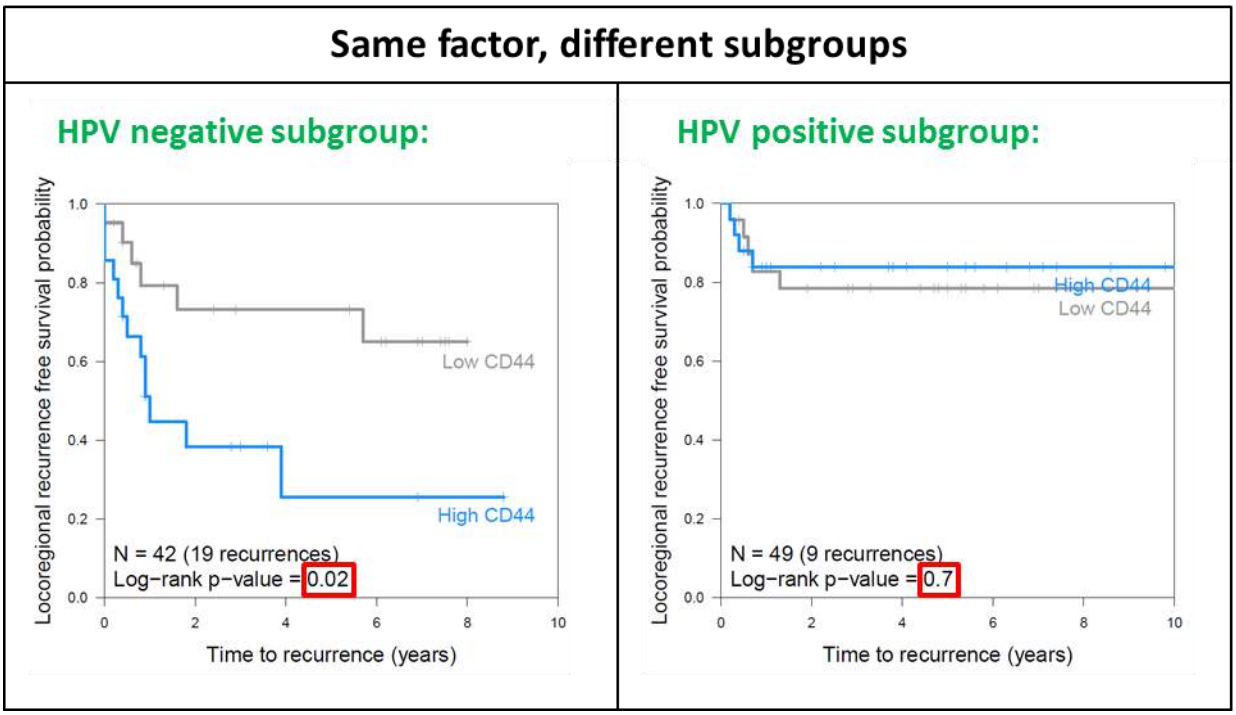

Figure 6.3: The effect of high or low CD44 expression on locoregional recurrence in two subgroups: HPV negative tumors (left panel) and HPV-positive tumors (right panel).

Not only HPV and CD44 show an interaction, but many correlations and interactions exist between different factors know to influence response to radiotherapy. Assuming that all 18 clinical and biological factors as mentioned in the introduction (age, sex, hemoglobin, health, smoking, T-stage, tumor volume, N-stage, tumor subsite, grade, HPV status, hypoxia, repopulation, redistribution, intrinsic sensitivity, stem cells, microenvironment, energy metabolism) are of importance for the prediction of control after radiotherapy and we would have two levels for all of those factors, we could make 324 (18^2) different groups that could possibly all have their own response rates. Obviously, many of those 324 combinations would have the same cure rates, since there is only so much room to make subgroups between 0 and 100% response rates. However, two groups with the same response rates to radiotherapy could have very different reasons for their failures. Especially different biological reasons for failure would be important to distinguish, since they would most likely result in different proposed treatment adaptations. An overview of some of the interactions for the different biological processes thought to contribute to radiotherapy response can be seen in table 6.1.

		HPV	Hypoxia	EGFR signaling	Repopulation	Redistribution	Intrinsic radiosensitivity				Stem cells/ CD44	Microenvironment		Energy metabolism
							EMT/miR-203	ROS	DNA repair	Cell death		Angio/ vasculogenesis	Immune system	
HPV		x												
Hypoxia		(37, 38)	x											
EGFR signaling		(39–41)	(42–44)	x										
Repopulation			(44)	(44, 45)	x									
Redistribution						x								
Intrinsic radiosensitivity	EMT/ miR-203	(46)	(32, 47–49)	(28, 34, 50, 51)			x							
	ROS						(30)	x						
	DNA repair	(40, 52)	(53)				(54)		x					
	Cell death		(55)		(56)					x				
Stem cells/ CD44		(14, 57, 58)	(32, 59–62)	(14, 28, 34, 63)	(64)		(22–34)	(30, 32, 64–66)	(29, 67, 68)		x			
Micro-environment	Angio/ vasculogenesis		(69)				(70)				(70, 71)	x		
	Immune system	(41, 72)	(73)				(74–77)	(78)			(74, 77)	(79)	x	
Energy metabolism			(32, 80)				(32)	(32)			(32, 81)			x

Table 6.1. Interactions and correlations between different biological processes thought to contribute to radiotherapy response described in literature. Click here for hyperlinks to references: 14, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81.

6.2.4 Prognostic vs. predictive factors

Another reason why we cannot start the immediate improvement of head and neck cancer radiotherapy with the use of HPV-status, Chung expression profile, CD44 and miR-203 measurements, is that so far they are prognostic and not certainly predictive (yet). A prognostic biomarker only has the ability to foretell outcome (irrespective of treatment), while a predictive biomarker is able to separate responders from non-responders to a certain therapy, meaning it can help support treatment decisions (82). So far, there are few predictive markers for head and neck cancer. A few markers have been found using retrospective analyses on randomized trials comparing two treatment arms. In a trial of normal overall treatment time versus accelerated radiotherapy, patients with high EGFR expression benefitted from accelerated radiotherapy, while the acceleration added no benefit in the group of patients with low EGFR expression (83, 84). However, EGFR expression is not currently used to decide whether a patient should have accelerated radiotherapy. Another predictive marker was found in a trial of hypoxia modification and radiotherapy, only the patients with a high hypoxia gene expression showed an improvement upon addition of nimorazole to radiotherapy (85). This hypoxia profile is now evaluated in a large prospective study (86). Furthermore, there is progress in the discovery of predictive biomarkers for response to EGFR-inhibitors (87, 88).

Ultimately, prognostic markers can be turned into predictive markers if the right (targeted) therapy is available. If for example, a CD44 inhibitor would only improve radiotherapy if added when a patient has a high expression of CD44, it would be predictive.

6.3 Rubbish in, rubbish out *(quote Adrian C. Begg)*

A well thought-out research plan and the accrual of reliable data is of the greatest importance for the generation of relevant, replicable results. Many factors should be taken into consideration when studying response to radiotherapy on pre-treatment tumor material.

6.3.1 The pre-treatment sample

Heterogeneity and tumor percentage

Different parts of a tumor could consist of cells with different genetic characteristics and radiosensitivity that are not being detected when only sampling a small part of the tumor (89, 90). In our studies we have used conclusions from biopsies of several millimeters as a surrogate for a tumor of several centimeters. Had tumor heterogeneity been an enormous problem, we would not have been able to use pre-treatment biopsies for outcome prediction at all. However, it seems reasonable to assume that part of the information on the whole tumor is lost with this approach. Toustrup et al. tested how much information gets lost due to head and neck cancer heterogeneity by studying hypoxia gene expression in multiple (2-4) samples from 20 tumors (91). They showed that in 70% of the tumors all replicate samples were awarded the same hypoxia score. However, when only samples with the highest percentage of tumor cells were selected, only 10% of patients would have wrongfully been classified as having less hypoxia. This is another difficulty with tumor biopsies: it will mostly consist of both tumor cells and stroma, different percentages of these two in a studied biopsy might lead to different results. Roepman et al. conclude that there was a poor signature performance for a head-neck expression signature that predicts the presence of lymph node metastasis on samples that contain less than 50% tumor cells (92).

Monitor during treatment?

It is plausible that biology changes during treatment. Still, it appears that we are fairly capable of predicting the response to radiotherapy on a pre-treatment sample, for example **chapters 2 and 3**, ref. (20, 93) and many others. As shown in **chapter 4**, not the changes in gene expression after irradiation, but the baseline microRNA levels in unirradiated cells correlated with radiosensitivity. Similarly, we know that fast repopulation of tumors only starts around the fifth week of radiotherapy (94), but benefit from accelerated radiotherapy can be predicted on a pre-treatment sample (83, 84). However, we might miss some biological changes during treatment that would be useful to improve treatment by adaptation during therapy. A study taking multiple biopsies during treatment is hard to conduct and not very patient-friendly. Imaging modalities like MRI or PET are more

convenient to study biology during treatment and possibly adapt treatment for non-responders (95, 96), although the monitoring of multiple biological processes will be far more challenging. There have been some reports suggesting that a change in certain PET tracers early during a course of radiotherapy better predicts treatment outcome than only pre-treatment uptake values (97, 98, 99). However, the opposite has been reported as well (100). Another possibility would be to monitor biomarkers in saliva or blood (101, 102, 103, 104).

6.3.2 Just (messenger) RNA?

The studies in **chapter 2** **and** **3** have used just messenger RNA to study the active biological processes in a tumor. As mentioned in the introduction, just messenger RNA might not entirely depict what happens in a cell. Therefore, microRNAs were integrated in the analysis in **chapter 4**, and one of them was shown to be the most useful predictor of radiosensitivity. Perhaps this is a result of the absence of a correct messenger RNA set for the same process, or the fact that there is less degradation of microRNAs during sample-handling, but could just as well result from the fact that messenger RNA alone is not enough, as has been shown by Jung et al. By combining data on methylation, DNA copy number, messenger RNA and microRNA they were able to better select patients at risk for metastases that with any of those methods alone (105). Another possibility is that we lose information because of the complicated statistics involved in the analysis of gene expression data. For example, before the final analysis, all samples in **chapter 4** were normalized using the assumption that the total amount of microRNAs is the same in every sample, while there is evidence that levels of microRNA differ between samples (62, 106).
Ideally, all possible pre-treatment information for a large group of patients would be collected (DNA methylation, DNA and RNA sequencing, protein levels and their phosphorylation status, different CT/MRI/PET scans, blood and saliva parameters) to filter out the most useful biomarkers for different therapeutic approaches (107). But even with all this information, it remains crucial to know which markers reliably represent certain processes and how we can target these processes to improve radiotherapy.

6.3.3 Need for adequate biomarkers of processes

Critics of gene expression profiles argue that many gene sets are not ready for clinical use because of the large differences between reported sets in literature. Results are not reproducible and therefore not deemed useful (1). According to our data, this is partly based on the misconception that different sets of genes per definition classify patients differently. In **chapter 5** we show that for hypoxia different sets of genes have been reported, with almost no overlapping genes. However, almost entirely different sets of genes can come to the same conclusion. While this is true for hypoxia, there are other processes that are still lacking reliable methods to assess the absence or presence of a factor causing radioresistance. Another problem illustrated in **chapter 5**, is that while it was assumed by most authors that they were studying both acute and chronic hypoxia, the gene sets only corresponded with an *in vitro* chronic hypoxia profile, which has a different supposed origin (lack of perfusion and not diffusion) and could have consequences for the appropriate therapeutic intervention.

 6.4 How to individualize future treatment?

With the ability to assess the possible causes for radioresistance of a tumor on a pre-treatment sample, we would be able to allocate the best fitting radiotherapy schedule and biological agent combination, eventually leading to better survival and/or less toxicity. Therapeutic options consist, apart from surgery, of various radiotherapy doses and fractionation schedules, dose painting, as well as the addition of cisplatin, hypoxia sensitizers, EGFR-inhibitors, hopefully soon to be expanded with for example immune checkpoint- (108, 109), DNA repair- (110, 111) or CD44-inhibitors (112, 113). Having multiple therapy options is an asset, but only if we know when to use which treatment.

Data-driven analyses on small patient series to find prognostic gene sets are not the way forward. Preferably we should focus on finding predictive markers in (randomized) studies, controlled for known factors. To move forward to the point where we know exactly which patient should get which treament(-s) we would have to study complete clinical and biological data from large numbers of patients that have been treated with different treatment alternatives (114). Within such a large cohort it would be possible to study subgroups and interactions between different biological factors and come up with the best predictive model (1). Furthermore, a subgroup could possibly be isolated that does not respond to any of the available treatments. Knowing the biological profile of these tumors could help design new ways to improve their treatment outcome. To achieve this, a database should be set-up across multiple countries, possibly combining already available data, with the ability to add data from new trials.

6.5 References

(Hyperlinks to references in text)

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Summary

The average overall survival for head and neck cancer is around 50%, but can vary significantly between groups of patients with different characteristics. Currently only clinical characteristics are used and treatment choice (often including radiotherapy) is based on site and TNM stage, which explain only a small proportion of the variation in survival. The research presented in this thesis describes studies into the individual biological tumor properties of head and neck cancer, using messenger- and microRNA data to predict which tumors will be more radioresistant and to gain insight into the mechanisms behind this. Eventually this should lead to a better understanding of the causes for radiotherapy failure allowing an up-front adaptation of therapy to give each individual head and neck cancer patient the best chances of survival with the least amount of toxicity.

Chapter 1 gives a general introduction into head and neck cancer and reviews existing knowledge on reasons for failure of radiotherapy. The general aims and outline of this thesis are also described in this chapter.

The first question to be addressed was whether gene expression data could add useful information to known clinical factors in the prediction of outcome after (chemo-)radiotherapy for head and neck cancer. In **chapter 2** we show that gene expression can improve the prediction model and adds valuable information to known clinical factors for the prediction of local control after chemoradiotherapy for advanced head and neck cancer.

We analyzed pre-treatment gene expression data from 75 advanced head and neck cancer patients treated with primary chemoradiotherapy. In this series a published high risk signature (Chung high-risk) and a HPV expression profile (Slebos) were analyzed in a model with known clinical predictors of local control: age at diagnosis, gender, tumor site, tumor volume, T-stage and N-stage. Only tumor site (oral cavity vs. pharynx, hazard ratio 4.2 [95% CI 1.4–12.5]), Chung gene expression status (high vs. low risk profile, hazard ratio 4.4 [95% CI 1.5–13.3]) and HPV profile (negative vs. positive profile, hazard ratio 6.2 [95% CI 1.7–22.5]) significantly predicted local control after chemoradiotherapy in the multivariable model.

Chapter 3 describes the analysis of a more homogeneous series of patients, treated with single modality radiotherapy. The hypothesis was that this series would give a better insight into the cause of radioresistance, without confounding by heterogeneity or clinical factors.

Gene expression data were generated on pre-treatment biopsies of 52 T1-2 laryngeal cancer patients treated with radiotherapy. Since recurrence rates are low in this population, patients with a local recurrence were matched for T-stage, subsite, treatment, gender and age with non-recurrence patients (1:2). Gene sets for hypoxia, proliferation and intrinsic radiosensitivity did not correlate with recurrence, whereas high expression of the putative stem cell marker CD44 did (odds ratio 20.2 [95% CI 3.4-172.3]). Immunohistochemical analysis of CD44 expression on an independent validation series of 76 small laryngeal cancers confirmed CD44's predictive potential. For more insight into the function of CD44,

gene expression data of eight larynx cancer cell lines with known radiosensitivity were analyzed. In these cell lines, CD44 expression did not correlate with intrinsic radiosensitivity although it did correlate significantly with plating efficiency, consistent with a relationship with stem cell content.

In neither of the patient series in **chapter 2 and 3** published intrinsic radiosensitivity gene sets were significantly correlated with recurrence after (chemo-)radiotherapy. This was an unexpected finding, since it is known that for head and neck tumors the *ex vivo* measurement of radiosensitivity correlates with outcome after radiotherapy. It was therefore concluded that an accurate gene expression set correlating with intrinsic radiosensitivity in head and neck cancer was lacking.

Chapter 4 describes the search for an intrinsic radioresistance gene set. Having such a set would not only be helpful to predict sensitivity before start of treatment, but could also reveal biological processes that could be targeted to overcome intrinsic resistance. MicroRNA and messenger RNA expression was measured in irradiated and unirradiated samples of 32 head and neck squamous cell carcinoma (HNSCC) cell lines. Measurements on unirradiated cells correlated with resistance, whereas the response to radiotherapy seemed irrelevant for the prediction of resistance. The presence of epithelial-to-mesenchymal transition (EMT) and low expression of microRNAs involved in the inhibition of EMT were important radioresistance determinants. This finding was validated in two independent cell line pairs, in which the induction of EMT reduced radiosensitivity. For the most important microRNA (miR-203), downregulation strongly correlated with intrinsic radioresistance in cell lines and a higher recurrence rate after radiotherapy in a series of 34 laryngeal cancer patients.

In **chapter 5** we show that for hypoxia different sets of genes have been published, with almost no overlapping genes. However, almost entirely different sets of genes can come to the same conclusion. Four published gene sets were compared using expression data from 224 head and neck cancer patients from three different datasets. Although only 2% of all genes were similar in the four validated hypoxia profiles, the profiles showed a near complete correlation with each other in categorizing the 224 patients. While it was assumed by most authors that they were studying both acute and chronic hypoxia, the gene sets that were published only corresponded with an *in vitro* chronic hypoxia profile, not with the early hypoxia response profile. Additionally, this early hypoxia profile better predicted local recurrence after chemoradiotherapy.

Chapter 6 contains a general discussion of the work presented in this thesis. In this chapter possible pitfalls of the presented research are discussed. In the last part, directions for future research are explored.

Samenvatting

De gemiddelde overleving voor patiënten met hoofdhals kanker ligt rond de 50%, maar varieert sterk tussen verschillende groepen patiënten met verschillende eigenschappen. Voor het maken van behandelkeuzes worden momenteel alleen klinische eigenschappen gebruikt. De beslissing over welke behandeling gegeven moet worden, vaak onder andere bestaande uit radiotherapie, wordt gebaseerd op de locatie van de tumor en TNM stadiëring, eigenschappen die overigens maar een klein percentage van de variatie in overleving kunnen verklaren. Het onderzoek dat wordt gepresenteerd in dit proefschrift beschrijft studies naar individuele biologische eigenschappen van hoofdhals tumoren. Messenger- en microRNA data worden gebruikt om te voorspellen welke tumoren ongevoelig zijn voor bestraling en wat het mechanisme hier achter is. Uiteindelijk moet dit leiden tot een beter begrip van de oorzaken van resistentie tegen bestraling, zodat een behandeling hier van te voren op kan worden aangepast en hoofdhals kanker patiënten de best mogelijke overlevingskans hebben met zo min mogelijk toxiciteit.

Hoofdstuk 1 geeft een algemene beschrijving van hoofdhals kanker en een overzicht van de bekende factoren die kunnen bijdragen aan het falen van radiotherapie. De doelstellingen en hoofdlijnen van dit proefschrift worden ook beschreven in dit hoofdstuk.

De eerste te beantwoorden vraag was of gen expressie data iets kunnen toevoegen aan klinische factoren bij het voorspellen van de uitkomst van een behandeling met (chemo-)radiotherapie voor hoofdhals kanker. In **hoofdstuk 2** laten we zien dat de toevoeging van gen expressie data het voorspellen van de recidiefkans na behandeling verbetert en waardevolle informatie toevoegt aan de bestaande klinische factoren die gebruikt worden om een inschatting te maken van de kans op locale controle na chemoradiotherapie voor gevorderde stadia van hoofdhals kanker.

Gen expressie data gemeten vóór behandeling van 75 hoofdhals kanker patiënten met een gevorderd stadium behandeld met chemoradiotherapie werden geanalyseerd. In deze serie werden een gepubliceerd hoog-risico profiel (Chung high-risk) en een HPV expressie profiel (Slebos) geanalyseerd in een model met bekende klinische voorspellers van locale controle: leeftijd ten tijde van diagnose, geslacht, tumor locatie, tumor volume, T-stadium en N-stadium. Alleen tumor locatie (mondholte vs. farynx, hazard ratio 4.2 [95% CI 1.4–12.5]), Chung gen expressie status (hoog vs. laag risico profiel, hazard ratio 4.4 [95% CI 1.5–13.3]) en HPV profiel (negatief vs. positief profiel, hazard ratio 6.2 [95% CI 1.7–22.5]) waren significante voorspellers van locale controle na chemoradiotherapie in een multivariaat model.

Hoofdstuk 3 beschrijft de analyse van een meer homogene serie patiënten, behandeld met alleen radiotherapie. De hypothese was dat deze serie een beter inzicht zou geven in de oorzaak voor stralingsongevoeligheid, zonder ruis veroorzaakt door heterogeniteit of het effect van klinische factoren.

Gen expressie werd bepaald op biopten genomen voor start van de bestraling van 52 patiënten met een T1-2 larynxcarcinoom. Aangezien het recidiefpercentage laag is in deze populatie, werden patiënten met een locaal recidief 1:2 gematcht met patiënten zonder recidief voor de volgende factoren: T-stadium, locatie, behandeling, geslacht en leeftijd. Gen expressie profielen voor hypoxie, proliferatie en intrinsieke stralingsgevoeligheid correleerden niet met het krijgen van een recidief. Daarentegen was er een correlatie tussen de kans op recidief en een hoge expressie van vermeende stamcelmarker CD44 (odds ratio 20.2 [95% CI 3.4-172.3]). Met behulp van immunohistochemie werd deze bevinding in een onafhankelijke serie van 76 patiënten met kleine larynxtumoren gevalideerd.

Om meer inzicht te krijgen in de functie van CD44 werden gen expressie data van acht larynxcarcinoom cellijnen met een bekende gevoeligheid voor bestraling geanalyseerd. In deze cellijnen werd gezien dat CD44 expressie niet correleert met intrinsieke stralingsgevoeligheid, maar met plating efficiency, wat past bij een verband met kankerstamcellen.

In geen van de patiënten series in **hoofdstuk 2 en 3** waren eerder gepubliceerde gen sets voor stralingsgevoeligheid significant gecorreleerd met recidiefkans na (chemo-)radiotherapie. Dit was een onverwachte bevinding, aangezien het bekend is voor hoofdhals tumoren dat een ex vivo meting van stralingsgevoeligheid overeenkomt met recidiefkans na bestraling. Er werd daarom geconcludeerd dat er voor hoofdhals kanker geen adequaat gen expressie profiel bestond voor het voorspellen van intrinsieke stralingsgevoeligheid.

Hoofstuk 4 beschrijft de zoektocht naar een gen set voor intrinsieke stralingsgevoeligheid. Deze set zou niet alleen nuttig zijn voor het voorspellen van stralingsgevoeligheid voor de start van een behandeling, maar zou ook kunnen bijdragen aan het ontdekken van processen die gericht kunnen worden aangepakt om intrinsieke stralingsongevoeligheid op te heffen. MicroRNA en messenger RNA expressie werden gemeten in bestraalde en onbestraalde cellen. In het totaal werden 32 hoofdhals plaveiselcelcarcinoom cellijnen meegenomen in de analyse. De metingen in onbestraalde cellen correleerden met stralingsgevoeligheid, terwijl de gemeten respons op bestraling niet voorspelde welke cellen stralingsongevoelig waren. De aanwezigheid van epitheliale-naar-mesenchymale transitie (EMT) en een lage expressie van microRNAs die EMT inhiberen, waren belangrijke voorspellers van stralingsongevoeligheid. Deze bevinding werd bevestigd in twee onafhankelijke cellijn paren, waarin EMT werd geïnduceerd, leidend tot een verminderde gevoeligheid voor bestraling. Lage expressie van de belangrijkste microRNA (miR-203) correleerde sterk met intrinsieke stralingsongevoeligheid in cellijnen en tevens met een hoger recidief percentage na radiotherapie in een serie van 34 larynxcarcinoom patiënten.

In **hoofdstuk 5** laten we zien dat er verschillende sets van genen zijn gepubliceerd om hypoxie aan te tonen met haast geen overlappende genen tussen de verschillende sets. Niettemin komen deze zeer verschillende genen sets tot dezelfde conclusie. Vier gepubliceerde genen sets werden vergeleken met behulp van gen expressie data van 224 hoofdhals kanker patiënten uit drie verschillende datasets. Hoewel in de vier gevalideerde hypoxie profielen maar 2% van de genen in alle vier voorkwam, waren de onderlinge correlaties bij het categoriseren van de 224 patiënten erg hoog. De meeste auteurs gingen er van uit dat hun profiel een maat was voor acute en chronische hypoxie, maar de

gepubliceerde sets correleerden alleen met een *in vitro* gegenereerd chronisch hypoxie profiel en niet met een acuut hypoxie profiel. Bovendien voorspelde het acute hypoxie profiel beter welke patiënten een locaal recidief kregen na chemoradiotherapie.

Hoofdstuk 6 betreft een algemene discussie van het onderzoek beschreven in dit proefschrift. In dit hoofdstuk worden mogelijke tekortkomingen van dit onderzoek bediscussieerd. In het laatste gedeelte worden perspectieven voor toekomstig onderzoek besproken.

Curriculum vitae

The author of this thesis was born on August 29th, 1982 in Leiden, The Netherlands. In 2000 she graduated cum laude from the Bernardinuscollege (Gymnasium) in Heerlen and went to medical school in Utrecht. During her studies she became interested in cell biology, oncology and radiology and therefore did an internship at the department of Radiation Oncology of the Princess Margaret Hospital (Toronto, Canada). During this internship she learned about the existence of radiobiology and decided she wanted to become a radiation oncologist and radiobiologist. In the final year of her training she did a research internship in the group of prof.dr. Adrian C. Begg at the Netherlands Cancer Institute - Antoni van Leeuwenhoek. After obtaining her Medical Degree in 2007, she continued her research on gene expression profiles to predict radioresistance of head and neck cancer. This research was funded by a joint KWF (Dutch Cancer Society) project supervised by prof.dr. Adrian C. Begg, prof.dr. Michiel W.M. van den Brekel, dr. Frank J. Hoebers, prof. dr. Coen R.N. Rasch and prof.dr. Marcel Verheij. The research was combined with a residency at the department of Radiation Oncology at the same institute. On December 1st, 2017 she will finalize her radiation oncology residency (supervisors: drs. Joost L. Knegjens and dr. Astrid N. Scholten).

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Presentations (selection):

‘Can gene expression predict response to radiotherapy of head and neck cancer?’, proffered paper, ESTRO, September 2008.

‘Prediction of local recurrence after radiotherapy in head and neck cancer by expression profiling’, proffered paper, ICTR, March 2009.

‘Prediction of local recurrence after radiotherapy in head and neck cancer by expression profiling’, proffered paper award, Netherlands Society for Radiobiology, April 2009.

‘CD44 as a predictive marker for outcome after radiotherapy’, Staff meeting dept. of Experimental Therapy NKI-AvL, October 2009.

‘Potential predictors for radiosensitivity in head and neck squamous cell carcinoma’, invited speaker at OncoRay Dresden, September 2013.

‘Predictors of recurrence after radiotherapy in head and neck squamous cell carcinoma’, Staff meeting dept. of Radiotherapy NKI-AvL, April 2014.

‘MicroRNAs and radioresistance in head and neck cancer’, invited speaker, ESTRO, August 2014.

‘Hypoxia gene expression in head and neck cancer – same same but different’, CERRO, January 2015.

‘Gene expression to predict prognosis of head & neck cancer’, CERRO, January 2016.

‘Prognostic gene expression signatures in HNSCC’, invited speaker, Workshop Biomarkers for Radiation Oncology, OncoRay Dresden, May 2016.

‘The new ‘Rs’ in radiation biology’, teaching lecture, ESTRO, May 2016.

‘Genetic biomarkers: mRNA/miRNA profiles’, teaching lecture, pre-meeting course ESTRO, May 2017.

‘Stereotactic radiotherapy for oligometastases’, AvL Symposium, June 2017.

‘The radiobiology of radiosurgery’, invited speaker, Symposium 15 years Gamma Knife Center Tilburg, June 2017.

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Professor Adrian C. Begg †

February 12, 1946 – January 29, 2014

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Obituary
Obituary Adrian Begg ESTRO

Lectures given by Adrian Begg at the MAASTRO Clinic 2011/2012:
The Linear-Quadratic (LQ) Model – all you wanted to know but were afraid to ask
Flow cytometry: principles, and (mainly) cell cycle applications in radiation oncology
Good and bad ways to assess treatment response
Exploiting DNA repair to improve radiotherapy
Tumor proliferation: basic concepts and therapeutic possibilities

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