Chapter 1

General introduction and outline of the thesis.
General introduction

Epidemiology and Etiology

Head and neck cancer is a heterogeneous group of malignancies that may arise from different parts of the head and neck region, such as from the salivary glands or the skin. However, most malignant tumours (90%) in the head and neck region are squamous cell head and neck carcinomas (HNSCC) originating in the mucosa of the oral cavity, oropharynx, nasopharynx, hypopharynx and/or larynx. In this thesis, the focus will be on these HNSCC.

HNSCC is the sixth most prevalent type of cancer in the world[1]–[3], with an incidence of 9.2 patients per 100,000 people per year [4]. Risk factors for HNSCC are tobacco smoking, alcohol consumption, infection with the human papilloma virus (HPV) [5], [6] and chewing of the betel quid.

Cigarette smoke contains nitrosamine and polycyclic hydrocarbons, which are known to be genotoxic, and therefore carcinogenic. Heavy smokers have an approximately 2-fold increased risk for developing HNSCC as compared to non-smokers.[7] The combination of alcohol consumption and smoking has been shown to increase the risk even more, up to a 6 fold. The common idea is that alcohol partially dissolves the mucosa, leaving the epithelium more susceptible to the carcinogenic effects of the tobacco smoke.[7]

In the last decade it has become clear that the high risk HPV types play an important role in the carcinogenesis of HNSCC, mostly in the oropharyngeal region, and more specifically in the base of the tongue and tonsils. HPV related tumours demographically occur more in Caucasian, male, non-smoking, non-drinking, younger patients with a higher socio-economic status, when compared to non-HPV related HNSCC patients. Sexual behaviour is thought to be the most important risk factor in HPV related HNSCC[8]. HPV related HNSCC has a 2 times better overall survival than HPV unrelated HNSCC. [9]

Chewing of the betel quid or Paan is common in south-east Asia and certain regions of India. A betel quid consists of areca nut and betel leaves, sometimes with addition of tobacco, slaked lime (calcium hydroxide) paste is often added to bind the leaves. Both the betel leaves and the areca nut have carcinogenic effects[10]. Also it has been shown that the slaked lime makes the mouth more
alkaline, which is thought to increase the carcinogenic effects of the areca nut and betel leaves.[11]

**Treatment**

Patient with HNSCC can be treated with different strategies including primary surgery either followed or not by postoperative radiotherapy or chemo-radiation. Alternatively HNSCC patients can be treated with non-surgical strategies, such as primary radiotherapy either as single modality, combined with chemotherapy [12] or, targeted agents directed against the epidermal growth factor receptor (EGFR)[13]. In general, patients with early stages (stages I-II) have an excellent outcome and are most optimally treated with only one treatment modality (surgery or radiotherapy alone). It should be noted that there are no randomized controlled trials to confirm this, but the results in these cases in terms of loco-regional control and survival are favourable. Most patients with HNSCC however present with more locally advanced stages (stages III and IV). Although loco-regional tumour control and survival associated with non-surgical strategies have improved significantly over time[12], in particular after the addition of concurrent chemotherapy to radiotherapy, many patients with locally advanced HNSCC are still primarily treated with surgery and are referred for postoperative radiotherapy or chemo-radiation in case of adverse prognostic pathological factors. This is particularly true for oral cavity SCC. The choice between primary surgical or non-surgical treatment strategies depends thus on several factors such as primary tumour origin and expected functional outcomes. Besides considerations of loco-regional control and survival, the development of treatment-related side effects depends on both the selected treatment strategy and the site of the primary tumour. This, for example, results in an emphasis on surgery in cancers of the oral cavity and on non-surgical approaches in HNSCC originating from the larynx, hypopharynx and oropharynx.

Still many patients with locally advanced HNSCC are primarily treated with surgery of the primary site and neck dissection with or without postoperative radiation or chemo-radiation. Although there are no large randomized controlled trials (RCT’s) that confirm the additional value of postoperative radiotherapy after primary surgery as compared to surgery alone, it is generally believed that radiotherapy is effective against microscopic deposits of cancer cells which, if unaddressed, would progress and lead to tumour recurrence.
In a number of retrospective studies, the results obtained with surgery alone were compared to those obtained with surgery and postoperative radiotherapy[14]. In most of these studies, loco-regional tumour control was significantly better after surgery and postoperative radiotherapy compared to surgery alone for the entire population[14]. This was particularly true in case of positive surgical margins [15] and lymph node metastases with extranodal spread[15], [16]. In most of these series, the benefit in terms of loco-regional tumour control was associated with a significant overall survival benefit as well[14].

According to the Dutch guidelines, postoperative radiotherapy is currently considered standard in case of high risk factors after surgery alone, including positive (< 1 mm) or close (1-5 mm) surgical margins, lymph node metastases with extranodal spread, 2 or more positive lymph nodes, invasion of the soft tissues and/or skin of the neck, more than 5 mm subglottic extension and perineural growth [Dutch guidelines].

The patients included in the studies of this thesis were all treated with primary surgery and considered at risk for locoregional failure and treated with postoperative radiotherapy after primary surgery.

Prognosis after surgery and postoperative radiotherapy

Patients with HNSCC may present with a large variety of prognostic factors, including clinical factors, pathologic features and molecular and genetic characteristics. For the proper design of therapeutic trials investigating novel strategies, a thorough knowledge of differences in outcome of specific prognostic groups is essential. Moreover, the choice of adjuvant treatment (e.g., total dose, overall treatment time, radiotherapy technique, addition of chemotherapy) after primary surgery mainly depends on the risk on loco-regional failure and to a lesser extent on the probability of distant metastases.

Langendijk et al developed a classification system based on recursive partitioning analysis (RPA)[17]. Based on this RPA, three different risk groups among patients treated with surgery and postoperative radiotherapy could be identified. RPA Class I (intermediate risk) consisted of patients who had no pN3 lymph nodes, free surgical margins (> 5 mm), and no extranodal spread (ENS). RPA Class II (high risk) consisted of patients who had 1 positive lymph node with ENS or had pT1, pT2, or pT4 tumours with close or positive surgical margins. RPA Class III (very high risk)
consisted of patients who had a pN3 neck, > 2 positive lymph nodes with ENS, or a pT3 tumour with close or positive surgical margins. This classification system was significantly associated with outcome in terms of loco-regional tumour control, overall survival and the occurrence of distant metastases. The prognostic value of the RPA classification system was externally validated in two other studies [18], [19]. The RPA risk groups may be used to select the most appropriate adjuvant therapeutic strategy after curatively intended primary surgery.

Besides clinico-pathological factors, there are a number of other factors directly related to treatment itself that may influence outcome after primary surgery and postoperative radiotherapy, including the interval between surgery

<table>
<thead>
<tr>
<th>Variable</th>
<th>RPA class</th>
<th>Class I (intermediate risk)</th>
<th>Class II (high risk)</th>
<th>Class III (very high risk)</th>
<th>Log-rank p-value (DF = 2)</th>
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<tbody>
<tr>
<td>Locoregional control</td>
<td></td>
<td>92</td>
<td>78</td>
<td>58</td>
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<td>5-yrs (%)</td>
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<td>2.37</td>
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<td></td>
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<tr>
<td>95%CI</td>
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<td>1.43–3.94</td>
<td>3.22–8.62</td>
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<tr>
<td>Distant metastases free interval</td>
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<td>80</td>
<td>68</td>
<td>&lt; 0.0001</td>
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<tr>
<td>5-yrs (%)</td>
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<td>2.48</td>
<td>4.74</td>
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</tr>
<tr>
<td>95%CI</td>
<td></td>
<td>—</td>
<td>1.44–2.28</td>
<td>2.77–8.13</td>
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<tr>
<td>Disease-free survival</td>
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<td>47</td>
<td>32</td>
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<td>1.54</td>
<td>2.48</td>
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</tr>
<tr>
<td>95%CI</td>
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<td>—</td>
<td>1.20–1.97</td>
<td>1.93–3.20</td>
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<td>51%</td>
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<td>—</td>
<td>1.64–3.84</td>
<td>3.14–7.29</td>
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<td>Overall survival</td>
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<td>5000%</td>
<td>36</td>
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<tr>
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<td>—</td>
<td>1.56–1.91</td>
<td>1.81–3.04</td>
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</tr>
</tbody>
</table>

RPA: recursive partitioning analysis; DF: degrees of freedom; HR: hazard ratio; 95%CI: 95% confidence interval
and postoperative radiotherapy, the overall treatment time of postoperative radiotherapy and the total radiation dose administered[14]. The outcome after surgery and postoperative radiotherapy in the different risk groups are depicted in Table 1.1[14].

*Molecular and genetic prognostic factors*

In order to identify molecular markers for clinical outcome in HNSCC, we have selected a number of proteins known to have aberrant expressions in HNSCC, or known to be of clinical significance in other types of cancer.

The Epidermal Growth Factor Receptor (EGFR) is member of the Human Epidermal Growth Factor Receptor (HER) protein family, which consists of 4 member proteins (HER1 – 4)[20], [21]. The HER proteins are membrane receptor tyrosine kinase proteins. EGFR responds to extra-cellular signalling molecules such as EGF and TGFα, by dimerization and auto-phosphorylation, which triggers a cascade of phosphorylations in the PI3K/PTEN/AKT pathway, the RAF/MEK/ERK pathway and the JAK/STAT pathway, leading to increased proliferation[20], [22]–[24]. EGFR has been described in multiple types of cancer to have an effect on the response to treatment. EGFR is found to be mutated[25], [26], amplified[27], alternatively spliced [28], [29] or overexpressed [30], [31] resulting in EGFR activation and in all cases associated with a negative effect on clinical outcome. Also multiple aberrations are found in the downstream members of this signalling pathway. Amongst these are activating mutations in activating members of the pathway such as the catalytic subunit of PI3K (PIK3CA)[23], [32], [33], deletion of the signal inhibiting PTEN protein[34]–[36].

Chromosomal aberrations are a common event in most types of cancers. They consist mostly of amplification or deletions of certain regions of the genome. The frequency of chromosomal aberrations in HNSCC has been studied previously[37], [38], and the amplification of the 11q13 region occurs most frequently in HNSCC (approximately 36%). Previously the amplification of this region has also been described for breast (approximately 20%)[39], [40] and ovarian cancer (approximately 16%)[41]. The size of the 11q13 amplicon is not identical for all tumours with the amplification. Within the amplicon a core can be identified, i.e. the region that occurs most frequently in the amplicon[37], [38]. Within the core of the 11q13 amplicon, in HNSCC 13 genes are identified and of these 13 genes, amplification of the FADD gene associated best with increased mRNA
expression[38].

The Fas Associated Death Domain containing protein (FADD) is best known for its role in the extrinsic apoptosis pathway, where it functions as an adaptor protein between the Fas receptor (FasR/CD95/TNFRSF6), and procaspase-8. Upon activation of the Fas Receptor by the Fas Ligand (FasL), the Fas Receptor will cluster and attract the members of the Death Including Signalling Complex (DISC), which, through caspase 3 and PARP will result in cell death[42]–[44]. The FADD protein consists of 3 domains: a Death Domain (DD) which binds to the DD in Fas Receptor, a Death Effector Domain (DED) which binds to the DED in Caspase 8, and a C-terminal domain[45]. Within the C-terminal domain a location was identified where FADD can be phosphorylated (Ser194). The phosphorylation at Ser194 has an effect on cell cycle regulation[43], [46]–[50].

Serine 194 phosphorylated FADD (pFADD) is present almost exclusively in the nucleus of the cell, where it co-locates with the mitotic spindles during the metaphase of the cell cycle [48]–[51]. Furthermore, arresting the cell in the G2/M phase of the cell cycle by treatment with Taxol resulted in higher levels of pFADD. [49], [52]

The protein expression patterns[53]–[56] and genetic make-up of a tumour, e.g. mutations[53], [57], [58] and genomic aberration[59]–[61], are of a large influence on the clinical outcome after treatment. In order to find prognostic markers for the clinical outcome in HNSCC, we have studied the expression of a number of proteins and associated the expression of these proteins to various forms of clinical outcome of the patients.

Goal and Hypothesis

The goal of the research project described in this thesis was to improve outcome prediction for squamous cell carcinoma of the head and neck, when treated with primary surgery and postoperative radiotherapy. We attempted to do this by using protein expression patterns in the tumour as candidate prognostic factors for overall survival, local or loco-regional control and distant metastasis. These proteins are referred to as molecular markers. Furthermore we studied the mechanisms behind the prognostic effect of these molecular markers on a cell biological level. This was done by overexpression in cell lines and performing in-vitro assays. We hypothesized that using both molecular markers and clinico-
pathological characteristics will give a more complete picture of the tumour, and will therefore result in a more accurate prediction of clinical outcome. More accurate clinical prediction models may also be important for treatment selection in individual patients, resulting in improvements of clinical outcome, and/or reductions in unnecessary adverse effects of treatment.
Outline of this thesis

To achieve this goal, several studies were undertaken, to find new markers that would lead to better prediction of clinical outcome, and eventually to improve the choice of treatment.

Chapter 2 “The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) mediates radiosensitivity in head and neck cancer” describes the effects of overexpression of proteins in the EGFr pathway on clinical outcome, and specifically on loco-regional recurrence. Additionally, the effect of PTEN overexpression on the sensitivity of this cell line upon in vitro radiation was investigated.

Chapter 3 “FADD expression is associated with regional and distant metastasis in Squamous Cell Carcinoma of the Head and Neck” describes the effects of overexpression of the Fas Associated Death Domain (FADD) on the metastatic behavior in a patient group of locally advanced squamous cell carcinoma of the head and neck.

Chapter 4 “FADD Expression as a Prognosticator in Early-Stage Glottic Squamous Cell Carcinoma of the Larynx Treated Primarily With Radiotherapy” describes the effects of FADD and phosphorylated FADD overexpression on local control in a patient series of early stage glottic carcinoma that were treated with radiotherapy alone.

Chapter 5 “High expression of phosphorylated Fas Associated Death Domain containing protein (pFADD) is associated with better local control in oral cancer” describes the effects of phosphorylated FADD expression on local control in a group of locally advanced Oral Squamous Cell Carcinoma. Also the effect of radiation sensitivity in a cell line overexpressing a phosphorylation mimicking form of FADD was described in this study.

Chapter 6 “Summary, general discussion and perspectives” will give an overview of the studies that have been performed in this thesis, and discuss where we should go from here.