Chapter 6
Summary, general discussion and perspectives
Summary

The goal of the studies described in this thesis was to improve clinical outcome prediction for squamous cell carcinoma of the head and neck (HNSCC) in two different treatment cohorts. The first cohort consisted of locally advanced HNSCC from various locations in the head and neck region that were treated with primary surgery and postoperative radiotherapy (POST-cohort). The second cohort consisted of early-stage (T1-2) glottic larynx SCC treated with definitive radiotherapy alone (GL-cohort).

In this thesis, expression of two major groups of proteins that are commonly deregulated in HNSCC were studied: (1) those in the genomic 11q13.3 region commonly amplified in HNSCC [37], and those involved in the EGFR pathway [156], [157]. To validate a possible direct relationship between expression of candidate genes and radiosensitivity, we expressed the genes that were found to be associated with response to radiotherapy or locoregional tumour control, in cell lines to determine in vitro cell survival upon irradiation.

In chapter 2 we studied the EGFR pathway in our POST-cohort. The aim was to identify proteins in this pathway with the strongest association with locoregional tumour control. The hypothesis was that increased activation of the EGFR pathway would be associated with worse locoregional control, since the EGFR pathway promotes survival [30], [158], [159]. In this analysis, we investigated the impact of proteins that indicate an activated EGFR pathway (i.e. pEGFR, pAKT and pERK) and other proteins in the pathway of which the expression could modify this activation (EGFR, HER2, PI3K and PTEN). EGFR [156], [159], [160] and HER2 [161], [162], both positive regulators of the pathway, are often amplified in various tumours. Moreover, the PI3K subunit PIK3CA, often has activating mutations [156], [163], [164], while PTEN, is often deleted [163]. However, we found that PTEN expression positively correlated with the presence of pAKT, and that PTEN expression was associated with worse locoregional control. As PTEN is an inhibitor of the EGFR pathway, we expected that overexpression of PTEN would result in less AKT activation (pAKT), and that overexpression of a known tumour suppressor gene like \textit{PTEN} in a tumour would result in better clinical outcome. Recently, other investigators found an alternative function for PTEN in which PTEN interacted with the Rad51 and CENP-C proteins [165], [166] instead of inhibiting the EGFR pathway. CENP-C stabilizes centromeres, while Rad51 repairs double
strand breaks in the genome [165], [167]. Particularly, the interaction of PTEN with Rad51 could be relevant, since one of the major reasons for cell death after radiation is the presence of double strand breaks in the genome [168]. Stimulation of a double strand break repair protein would explain an increased resistance to radiation [168]. To test this, we overexpressed PTEN in a cell line, and found indeed that increased PTEN expression resulted in increased Rad51-expression in the cells. The overexpression of PTEN in these cells resulted in increased radiation resistance as measured by a clonogenic radioresistance assay. The association we found in the clinical data, between the overexpression of PTEN, and a worse local control, could therefore be explained by an improved double strand break repair mechanism in the tumour cells, through the association between PTEN and Rad51.

In chapters 3, 4 and 5 we studied proteins associated with the 11q13.3 region of the genome, which is the most frequently amplified genomic region in oral squamous cell carcinoma (OSCC). In this region the Fas Associated Death Domain (FADD) gene is overexpressed upon DNA amplification [38]. FADD plays a role in both (TNFR-mediated) apoptosis and when phosphorylated on Ser194 (pFADD) in regulation of cell cycle progression[155].

In chapter 3 we studied the prognostic value of FADD expression in a group of 177 HNSCC patients from our POST-cohort. FADD expression was assessed on the surgically removed tumour tissue using immunohistochemistry. High FADD expression was detected in 44% of the HNSCC patients. High expression was associated with an increased rate of lymph node metastases (p=0.001) and with a shorter distant metastasis free interval (DMFI) (HR: 3.3, 95%CI: 1.3-8.1, p=0.008) independent of lymph node status at the time of diagnosis. These data indicate that the expression of FADD in locally advanced HNSCC could be a valuable molecular marker for the potential of distant metastasis.

In chapter 4 the expression of FADD and pFADD was studied on 92 patients of the GL-cohort. The main focus of this study was to investigate the prognostic value of FADD and pFADD on local control. High levels of pFADD were associated with a significantly better local control (HR 2.403, 95% CI 1.041-5.548, p=0.040) while FADD overexpression showed a trend towards better local control (HR 3.656; 95% CI: 0.853-15.663, p=0.081). The multivariable analysis showed that pFADD was a better predictor for local control than other clinico-pathological factors, and that its prognostic value was independent of FADD expression. Although the
expression of FADD and pFADD was associated with local control, there was no association between this expression and overall and disease specific survival.

In chapter 5, the prognostic value of pFADD expression on the clinical outcome of oral SCC (OSCC) and its possible role in in vitro radiosensitivity was investigated. Surgically resected tumour tissue of 100 OSCC patients from the POST-cohort, were selected and stained for pFADD using immunohistochemistry. The sensitivity for radiation was validated in vitro in transfectants with regulated pFADD overexpression using the Grenman clonogenic cell survival assay. High expression of pFADD was a strong prognostic factor for better local control in OSCC (HR: 5.3; 95%CI: 1.6 – 18.0; p=0.007) which was confirmed in the multivariable analysis. Cells overexpressing pFADD proved to be more radiosensitive compared to control cell lines. The increased radiosensitivity in cells that overexpressed pFADD is in line with the association between pFADD and local control in OSCC. Thus, our data show that high pFADD expression is associated with increased radiosensitivity and better local control after primary surgery followed by postoperative radiotherapy.
General discussion

FADD expression is associated with lymph node metastases: is FADD expression also directly responsible for the metastatic potential?

FADD was initially described as an adapter protein involved in apoptosis [45]. So far, there were no previous reports suggesting that FADD expression was associated with the presence of lymph node metastases. In our POST-cohort we observed a significant association between high FADD expression and the presence of lymph node metastasis at diagnosis (Chapter 3), whereas in our GL-cohort no association was found between lymph node metastasis and FADD expression (Chapter 4). One explanation for the contradicting results between these two cohorts is the location in the head and neck region from which the tumour originated. Most HNSCC are well vascularized, a minimal requirement for tumour cells to metastasize to regional lymph nodes and tissues at distant locations [169], [170]. In our POST-cohort, where most HNSCC originated from the oral cavity (57%) and oropharynx (16%) there was indeed a high risk of lymph node metastases (64%). However, SCC that originate from the glottic larynx generally have a low rate of lymph node metastases, because of the limited lymph drainage system in the laryngeal region [133]. Moreover, patients with early stage (T1-2) tumours generally have low rates of lymph node metastases. This is clearly reflected in our GL-cohort showing low rate of lymph node metastases (2%). Consequently, the association between lymph node metastases and FADD expression in this GL-cohort (Chapter 4) could not be studied because of the small numbers of patients with lymph node metastases. In this respect, it should be emphasized that since the low rate of lymph node metastases in early stage glottis cancer, tumour markers that predict the presence of lymph node metastases are less relevant and might be particularly relevant for HNSCC at other locations with higher risks of lymph node metastases.

During the process of lymph node metastasis, cells from the primary tumour detach from its surrounding, and migrate through the lymph drainage system to the lymph nodes. These tumour cells then settle in the lymph node and proliferate again forming a tumour mass [169], [170]. The frequency of lymph node metastasis is dependent on a number of factors. The tumour cells need to have decreased interactions with the surrounding environment of both adjacent cells and the extracellular matrix (ECM), enabling easy detachment from the surrounding tissue[171]. To survive the migration from the primary tumour to the lymph
nodes, the tumour cells need to be insensitive to apoptosis upon detachment from the ECM, a process called anoikis, meaning “the state of being without a home”[172]. Another important factor is the abundance of lymph vessels through which the cells can migrate. These vessels are more present in some locations in the head and neck region (e.g. oral cavity), then in others (e.g. larynx). Therefore, oral cavity tumours more often develop lymph node metastases than laryngeal tumours [169], [170].

In our POST-cohort, we did not only observe a significant association between high expression of the FADD protein and the presence of lymph node metastasis at diagnosis (p<0.001), but also found an association between FADD expression and Distant Metastasis Free Interval (DMFI) (HR: 3.3, 95%CI: 1.3-8.1, p=0.008) (Chapter 3): To validate whether the association between FADD expression and both lymph node metastases and distant metastasis free interval directly resulted from expression changes of the FADD protein, we overexpressed a wild-type full-length FADD gene construct in HNSCC and other cell lines. However, our attempts to overexpress wild-type FADD failed in all these cell lines as FADD-transfected cells did not survive. This is in good agreement with the lack of studies reporting on continuous overexpression of the full-length FADD. In the TNFR apoptosis pathway, FADD functions as an adaptor protein, binding to the Fas receptor on the one side, and Caspase 8 on the other. There are two domains in the FADD protein that are necessary for this adaptor function. The N-terminal end of the protein contains a Death Effector Domain (DED), which binds to the DED of Pro-caspase-8, while the C terminal end has a Death Domain (DD) which binds to the DD of Death Receptors (e.g. TNFR and Fas). Studies that show a long term overexpression of FADD either only use the C terminal part of FADD (C-FADD)[47], [49]–[51], [155], which lacks the DED, or a dominant negative isoform of FADD (DN-FADD) with a mutated DED is mutated so that it cannot cluster with other DEDs anymore[173], [174].

Previous studies showed that overexpression of Death Effector Domain (DED) containing proteins such as FADD, resulted in the initiation of a clustering of these proteins into so called Death Effector Filaments [154]. These filaments can trigger a ligand independent, caspase dependent form of apoptosis, which means that the cells will go into programmed cell death through the caspase pathway, without receiving an extracellular trigger. These findings may explain the profound cell death that we observed in our wild-type full-length FADD overexpressing cell
lines. Assuming that this is true, the following questions remain to be answered regarding our observations in tissue samples:

1) Which mechanisms may explain the association between FADD and the metastasizing phenotype of tumour cells?

2) If overexpression FADD in our cell lines causes apoptosis triggered by Death Effector Filaments, why does this not happen in tumours overexpressing FADD?

In general, overexpression of wild-type FADD leads to apoptosis (Figure 6.1A) and when tumour cells start to migrate and invade through the basal membrane, they will normally die of anoikis (Figure 6.1B). However, if either of these two events occur in tumour cells with reduced sensitivity to caspase dependent apoptosis (Figure 6.1C1) and overexpression of FADD (Figure 6.1C2), cell migration will not lead to anoikis (Figure 6.1C4). Therefore, tumours with a reduced sensitivity to caspase dependent apoptosis are more likely to have high FADD expression and consequently are more likely to survive cell migration, resulting into increased lymph node and distant metastasis. This implies that FADD expression does not directly cause a metastasizing phenotype, but a third independent factor, the reduced sensitivity to caspase dependent apoptosis, which causes both the FADD expression and the metastasizing phenotype. This is known as a spurious relationship in which apoptosis sensitivity is the confounding factor. To prove that this is really the case, further studies should be performed in which FADD overexpression success rates and anoikis sensitivity are both measured in a background of Caspase inhibitors.

*pFADD expression is associated with local control: does pFADD expression directly affect radiosensitivity?*

The FADD protein can be phosphorylated at the serine on position 194, resulting in an isoform of the protein called phospho-Serine194 FADD, or pFADD. It has been reported that FADD is not only involved in apoptosis, but more recently that the pFADD isoform is involved in cell cycle regulation [49], [155]. In this thesis, we investigated the effect of a higher percentage of pFADD positive cells using primary tumours present in both the GL-cohort (Chapter 4) as in the POST-cohort (Chapter 5). In both cohorts we found that a higher percentage of pFADD positive cells in tumours was significantly associated with better local control (HR 2.4, p = 0.04 in the GL-cohort and HR 5.3, p = 0.007 in the Oral SCCs of the POST-cohort).
Figure 6.1 Illustration of the suggested link between FADD expression and metastasis through reduced sensitivity for Caspase dependant apoptosis. Six cell types are shown: = normal squamous cell; = normal cell in the basal cell layer; = tumour cell; = tumour cell overexpressing FADD; = tumour cells in apoptosis; = tumour cell with reduced sensitivity to caspase dependant apoptosis. A. Some tumour cells overexpress FADD (A2) and go into caspase-dependant apoptosis through formation of Death Effector Filaments (DEF) (A3). B Tumour cells start migration through the basal membrane (B2) and go in to caspase dependant apoptosis through anoikis (B3). C. Tumour cells first acquire resistance to caspase dependant apoptosis (C1) start overexpressing FADD which is usually an early event in carcinogenesis (C2) and survives that because Death Effector Filaments cannot trigger apoptosis (C3) then the cells start metastasizing which now does not trigger anoikis (C4).
To validate whether and how pFADD expression directly affects radiosensitivity in vitro, we constructed a number of stable cell lines containing FADD-gene constructs under control of an inducible promoter. Since it is not possible to express a specific non-phosphorylated or phospho-isoform of a protein, we used two different mutant FADD genes [48]–[50]. Both mutants contained a mutation in the triplet encoding for the Serine on position 194, in the first it changes to an alanine (FADD-S194A) and in the second to a glutamic acid (FADD-S194E). The FADD-S194A cannot be phosphorylated, and is therefore used as the non-p-FADD, whereas the FADD-S194E mimics pFADD, since the protein will be expressed with a bulky negatively charged group at the position of Serine 194, similar to a phosphate group.

Using cell lines with these 2 inducible FADD-isoforms, our in vitro clonogenic radiation resistance assays showed a worse cell survival of cells overexpressing FADD-S194E when compared to cells without expression. The effect on radiosensitivity of the phospho-mimicing FADD isoform was specific because the same cell line transfected with the FADD-S194A construct or the empty vector control had no effect on cell survival upon irradiation.

In primary tumours, pFADD was found mainly in the nucleus and is highly expressed during the G2/M phase of the cell cycle. In two HNSCC cell lines we demonstrated that arresting these cells in the G2/M phase of the cell cycle showed indeed increased expression of pFADD. We studied the cell cycle distribution of cells overexpressing FADD-S194E, to determine whether phosphorylation of FADD is part of the mechanism by which cells are arrested in the G2/M phase. However overexpressing FADD-S194E did not lead to a higher fraction of cells at G2/M. Because cells in the G2/M phase of the cell cycle have been shown to be more radiosensitive [150], [151], our data suggest that the observed association with better local control in patients treated with radiotherapy cannot be explained by an increased numbers of cells in G2/M as the result of pFADD expression.

As mentioned before, in our primary tumours, pFADD is mainly expressed in the nucleus, as can be seen when using a specific antibody against the phosphorylated FADD isoform. Also in other tumours pFADD was found to be expressed in the nucleus[46], [51]. Because there was no antibody available to specifically detect unphosphorylated FADD only, to detect FADD expression in our 2 cohorts we used an antibody that detects both FADD and pFADD. Combining both antibodies, the unphosphorylated FADD isoform is mainly expressed in the cytoplasm although
we cannot exclude that it is also located in the nucleus together with pFADD. Expression of the FADD-S194A mutant induces a significantly higher transport rate to the nucleus, whereas the expression of FADD-S194E showed an only moderate amount of transport into the nucleus (Figure 5.7). However, despite the moderate amount of FADD-S194E that was translocated to the nucleus, the levels were apparently sufficient for the effect on cell survival after radiation. These data suggest that under normal physiological conditions FADD is transported to the nucleus in its un-phosphorylated form, and subsequently becomes phosphorylated in the nucleus. In our model, it might be that the phospho-mimicking FADD isoform is not efficiently transported to the nucleus for this reason.

In conclusion, our data indicate that expression of pFADD has a positive effect on the radiosensitivity of tumour cells. This increased radiosensitivity could explain the association between pFADD expression and better local control in patients with HNSCC treated with radiotherapy. The exact pathway that is effected by pFADD in regulating radiosensitivity is presently unknown and subject of further research.
Future perspectives

The use of molecular markers for prognostic purposes is an important first step in utilizing the full potential of these markers.

Through improvement of predicting clinical outcome in terms of locoregional tumour control and overall survival using molecular tumour markers, we might select a subset of patients with a fair chance of treatment success, or, vice versa, to consider alternative treatment strategies for those with poor outcome. However, expression of molecular tumour markers may also indicate mechanisms explaining why certain treatment modalities will or will not be of benefit for specific patients, or why certain treatment strategies are not successful. The latter is specifically important as poor outcome after definitive radiotherapy does not necessarily mean that other treatment strategies like primary surgery, will be more successful, e.g. in case molecular markers identify a more aggressive phenotype rather than specific resistance to ionising radiation.

From this point of view, another opportunity for the clinical utility of molecular markers is the so-called targeted approach. Here a tailored drug is developed against a certain molecular marker, which can either be a protein that is overexpressed, or a specific mutation in a gene. In most cases this marker then directly becomes the predictive marker for these therapies. The development and clinical testing of tailored drugs requires thorough understanding of how proteins interacts with other proteins and pathways in the tumour cell, and how the expression of this protein affects tumour progression and thus requiring clinical validation in randomized controlled trials. A better understanding of the tumour cell biology is essential for this type of approach. At present, targeted therapies are applied in many cancer types, such as in breast cancer (Herceptin in HER2neu-positive tumours) and metastatic melanoma (vemurafenib in tumours with a BRAF V600E mutation).

One of the issues with the use of molecular markers is the lack of understanding of the interaction between these markers. There are many studies indicating the one or the other marker as being prognostic for a specific type of treatment, but what would really be useful would be a more matrix like approach, showing the predictive, or prognostic value of a combination of a large number of markers. By this it would be possible to test a biopsy for these markers, and select the appropriate treatment on basis of the combination of these markers. With new
advances in molecular biology and sequencing technologies, in the near future it might be possible and affordable to screen the tumours of thousands of patients for thousands of loci on the cancer genome. When linking this information to the treatment and clinical outcome information, predictive algorithms could be developed, on basis of which the best treatment for new patients could be chosen.

This approach could well be combined with the ex-vivo culturing of tumour in so called organiods[175]–[177]. In this method tumour and supporting tissue are cultured in a 3D matrigel, by which a high percentage of tumours can be cultured ex-vivo (~90%)[175]–[177]. The tumour tissue can be sequenced, expression profiled, and can undergo a high throughput drug screening, by which the sensitivity to many different potential drugs can be established. The sensitivity to these drugs can be used for a highly personalized treatment of the patient and additionally this information can be used to predictive algorithms, as described before.

The combined knowledge of genomic sequence, expression profiles, drug screening outcome, clinical treatment and clinical outcome will lead to a very powerful tool to tailor treatment and improve the clinical outcome in cancer patient in the near future.