Radiosensitivity and metastasis in squamous cell carcinoma of the head and neck

Pattje, Wouter Johannes

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Chapter 5

High expression of phosphorylated FADD is associated with better local control in oral squamous cell carcinoma patients and increased in vitro radiosensitivity.

Wouter J. Pattje\textsuperscript{1,2}, Lorian Slagter-Menkema\textsuperscript{1,3}, Mirjam F. Mastik\textsuperscript{1}, Michiel L. Schrijvers\textsuperscript{1,3}, Johan H. Gibcus\textsuperscript{1}, Bernard F.A.M. van der Laan\textsuperscript{3}, Jan L.N. Roodenburg\textsuperscript{4}, Olga Hoegen-Choevalova\textsuperscript{2}, Jacqueline E. van der Wal\textsuperscript{1}, Johannes A. Langendijk\textsuperscript{2\&}, Ed Schuuring\textsuperscript{1\&}.

1. Department of Pathology, 2. Department of Radiation Oncology, 3. Department of Otorhinolaryngology/Head and Neck Surgery, 4. Department of Oral \\& Maxillofacial Surgery, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands.

\& Author contributed equally

Submitted.
Abstract

Background

The most frequently amplified genomic region in oral squamous cell carcinoma (OSCC) is chromosome 11q13.3 region. In this region the Fas Associated Death Domain (FADD) gene is overexpressed upon DNA amplification. FADD plays a role in both (TNFR-mediated) apoptosis and when phosphorylated on Ser194 (pFADD) in regulation of cell cycle progression. The aim of this study was to investigate the prognostic value of pFADD expression on the clinical outcome of OSCC and its possible role in in vitro radiosensitivity.

Methods

Surgically resected tumor tissue of 100 OSCC patients treated with primary surgery and postoperative radiotherapy, of which clinico-pathological as well as follow-up data were available, were selected and immunostained for pFADD. The sensitivity for radiation was validated in vitro in transfectants with regulated pFADD overexpression using the Grenman clonogenic cell survival assay.

Results

High expression of pFADD is a strong prognostic factor for better local control in OSCC (HR:5.3; 95%CI: 1.6 – 18.0; p=0.007). In a multivariate analysis, pFADD expression is the strongest and most significant prognostic marker for local control. Cells overexpressing pFADD proved to be more radiosensitive compared to control cell lines.

Conclusions

High expression of pFADD in OSCC patients treated with primary surgery and postoperative radiotherapy is associated with a better local control. In vitro experiments showed an increased radiosensitivity in cells that overexpressed pFADD in good agreement with the association between pFADD and local control. Our data show that pFADD expression levels do not only identify OSCC patients that might benefit from postoperative radiotherapy but also play an important role in the response of tumor cells to radiation.
Introduction

Squamous cell carcinoma of the head and neck (HNSCC) is the 6th most prevalent type of cancer in the world[2]. Oral squamous cell carcinoma (OSCC) is the largest group within HNSCC[141] with an annual incidence of 3.8 per 100,000 people worldwide[4]. In the last decades, major advances have been made in the treatment of HNSCC resulting in significant improvement of disease-free and overall survival[142], [143]. Most OSCC patients present with locally advanced tumors with 5 year overall survival rates of approximately 50% [62] and locoregional recurrence rates of 20 to 40%[17], [19].

In order to further improve the clinical outcome of OSCC, e.g. by selecting patients for more or less aggressive treatment strategies, it is pivotal to identify these high risk patients. At present, in the postoperative setting, certain pathological markers such as positive surgical margins and the presence of extranodal spread are used to predict clinical outcome and to select patients for either adjuvant radiotherapy or chemoradiation. However, for a more individualized approach, factors that are able to predict outcome (prognostic factors) and those that can predict the added value of a certain treatment approach (predictive factors), such molecular markers, are needed[144].

In HNSCC multiple chromosomal aberrations may occur in high frequency including the amplification of the chromosomal region 11q13.3 (~36%)[38], [118]. The most commonly amplified gene in the 11q13.3 region is the Fas Associated Death Domain (FADD) gene[38]. FADD was first identified as an adaptor protein in the extrinsic apoptosis pathway linking the Fas death receptor to the initiator caspase 8[45], [145]. More recently, an isoform of FADD that is phosphorylated on the serine 194 (pFADD), mostly present in the G2/M phase of the cell cycle in the nucleus of normal cells and is located on the mitotic spindles during the meta- and early anaphase was described[48]–[51].

The clinical significance of pFADD expression was reported previously in breast, prostate and lung cancer. In lung cancer high pFADD expression was associated with worse overall survival[132], in prostate cancer high pFADD expression correlated with lower Gleason score, indicating a less aggressive tumor [52] and in breast cancer higher pFADD correlated with reduced prevalence of lymph node metastases[51]. Recently we found that a high expression of pFADD was significantly associated with better local control in 92 early stage (T1-T2) glottic laryngeal
squamous cell carcinoma patients treated with definitive radiotherapy[117].

The main purpose of this study was to test the hypothesis that high pFADD expression was associated with local control in a well-defined series of locally advanced OSSC treated with surgery and postoperative radiotherapy. We also tested in vitro the hypothesis that pFADD expression is involved directly in the response to radiation. For that purpose, the response to radiation of a cell line transfected with a phospho-mimicking FADD mutant under control of an inducible promoter was determined using the in vitro Grenman assay.

Methods

Patients and tissue

For this retrospective study we selected patients diagnosed with primary OSCC who were uniformly treated with primary surgery and postoperative radiotherapy at the University Medical Center Groningen in The Netherlands between 1993 and 2003. Clinical and histopathological data were collected with a follow up of at least 3 years.

The clinico-pathological features of the 100 patients included in this study, were reported previously[31], [146]. The majority of the patients included in this study were male (59%), had positive lymph nodes in the neck (N+; 60%) and presented with advanced stage tumors (Stage III-IV) (73%)(Table 5.1). Patients with previous other malignancies were excluded.

In total 94 of these patients underwent a neck dissection (94%). Postoperative radiotherapy was administered to all patients because of the presence of one or more adverse factors including positive surgical margins (64%), lymph node metastasis with extra nodal spread (28%), advanced stage (stage III-VI) (73%) and perineural growth (30%).

H&E staining was performed on formalin fixed paraffin embedded (FFPE) tissue of the surgically resected tumors. Histopathology of all carcinomas was revised by an experienced pathologist (JEvdW).

All patient tissues were coded. This study was performed according to the Code of Conduct for proper secondary use of human tissue in the Netherlands, as well as to the relevant institutional and national guidelines. Informed consent was given
Immunohistochemistry

Immunohistochemical staining was performed as described previously[38]. Briefly, 3 µm thick sections of FFPE tumor tissue were deparaffinized and rehydrated in a gradient series of alcohol. Antigen retrieval was performed by incubating overnight at 80˚C in 0.1 M Tris/HCl (pH 9.0). Endogenous peroxidase was blocked in a 0.3% H2O2 solution. For the pFADD staining the sections were subsequently incubated with the rabbit anti-pFADD monoclonal antibody (#2781, Cell Signalling Technologies, Danvers, MA, USA) 1:100 diluted in PBS for 1 hour, Horseradish Peroxidase (HRP) conjugated Goat anti Rabbit (GaRPO) immunoglobulin G (IgG) (1:100) for 1 hour, a HRP conjugated Rabbit anti Goat (RaGPO) IgG (1:100) for an hour, and treated with 3,3’-di-aminobenzidine (DAB) chromogen solution (Dako, Glostrup, Denmark) and counterstained with haematoxylin. For the scoring of the immunostaining of pFADD, the percentage of tumour cells with nuclear staining was determined. The ideal cut-off point for dichotomizing these data was 50% as determined by a ROC curve analysis for local recurrence. This cut-off of gave a sensitivity of 81% and specificity of 50% (data not shown). Percentages of positive staining above the cut-off level were considered as high expression, and those below as low expression of pFADD. All slides were scored independently by two observers without prior knowledge of clinical data. In case of discrepancies between the observers, cases were reviewed by an experienced pathologist and scored on consensus opinion.

Cell Culture and Transfections

The Hek293 cell line was cultured in DMEM (1 g l⁻¹ glucose) with 10% fetal bovine serum, 2 mM ultra-glutamine, penicillin and streptomycin (all purchased from BioWhittaker, Basel, Switzerland).

The pcDNA3-FADD-S194A and pcDNA3-FADD-S194E plasmids were kind gifts from Dr. Marcus E. Peter (The Ben May Institute for Cancer Research, University of Chicago) [12]. The pIND-FADD-S194A plasmid was constructed using high-fidelity PCR on pcDNA3-FADD-S194A and pcDNA3-FADD-S194E with overhanging primers containing restriction sites for HinDIII and BamHI and a cMyc tag (using primers AAGCTTATGGAGCAGAAGCTGATCTCCGAGGAGGACCTGATCGACCCGTTCCTG- GTGCTGCTGCACTC and GGATCCTCAGGCAGGTTCGAGGACCTGATCGACCCGTTCCTGAGGTC)
and cloning the product into pInd (Invitrogen, Life Technologies, Grand Island, NY, USA) using the BamHI and HinDIII restriction sites. The success of both the high fidelity PCR and the cloning was confirmed by sequencing the resulting pIND plasmids. The cells were co-transfected with pInd (either containing FADD-S194A, FADD-S194E or Empty Vector) and pVgRXR (Ecdysone inducible expression system by Invitrogen) using Fugene 6 reagent (Roche Applied Sciences, Almere, The Netherlands) as described by the manufacturer. After transfection, the cells were cultured on a selection medium of Zeocin (for pVgRXR, Invitrogen) and G418 (for pInd, Invitrogen). Single colonies were picked and cultured separately under selective pressure.

Out of 10 clones transfected with pIND-FADD-S194A and 10 clones transfected with pIND-FADD-S194E, the ones with no/very low FADD expression in the untreated cells and the highest expression after 24 hours of treatment with 5uM ponasterone A were selected for further experiments. In all tests, the expression of the transgene was stimulated 24 hours before the start of the experiments, using ponasterone A (in alcohol 100%) at a concentration of 5uM or 100% alcohol only as a negative control.

FACS Cell Cycle Analysis

Cell Cycle analysis was performed as previously[147]. Briefly, hypotonic DNA staining buffer (0.1% Sodium citrate; 0.3% Triton–x 100; 0.01% Propidium iodide, 0.002% Ribonuclease A) was added to the cells and mixed well. Acquisition was performed on the flowcytometer (Calibur, BD Biosciences, San Jose, CA USA).

FACS analysis for pFADD intensity

Cells were harvested and fixated using 4% paraformaldehyde. Membrane permeabilization was done using a 1% saponine buffer. Primary antibody specific for pFADD (#2781, Cell Signalling Technologies, 1:50 in 1%sarponine/2%BSA/PBS) was incubated at 4 °C overnight. Secondary antibody (A-21428 GaR-Alexa555, Invitrogen) incubation was applied at 4 °C for 1 hour. Afterwards the cells were suspended in a solution of 10 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for the DNA content staining. Acquisition was performed on a flow cytometer (LSRII, BD Biosciences).
### Table 5.1 Patient characteristics stratified by pFADD expression levels

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Low (n (%))</th>
<th>High (n (%))</th>
<th>Total (n (%))</th>
<th>p-value</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Median (range)</td>
<td>61.5 (25 - 90) yr</td>
<td>57.3 (24 - 84) yr</td>
<td>59.7 (24 - 90) yr</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>Female</td>
<td>25 (43)</td>
<td>17 (41)</td>
<td>42 (42)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 (57)</td>
<td>25 (59)</td>
<td>58 (58)</td>
<td></td>
</tr>
<tr>
<td><strong>T-stage</strong></td>
<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>T1</td>
<td>6 (10)</td>
<td>4 (10)</td>
<td>10 (10)</td>
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<tr>
<td>T2</td>
<td>14 (24)</td>
<td>16 (38)</td>
<td>30 (30)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>9 (16)</td>
<td>8 (19)</td>
<td>17 (15)</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>29 (50)</td>
<td>14 (33)</td>
<td>43 (43)</td>
<td></td>
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<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>N0</td>
<td>21 (36)</td>
<td>19 (45)</td>
<td>40 (40)</td>
<td></td>
</tr>
<tr>
<td>N+</td>
<td>37 (64)</td>
<td>23 (55)</td>
<td>60 (60)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>ns</td>
</tr>
<tr>
<td>I</td>
<td>2 (4)</td>
<td>2 (5)</td>
<td>5 (5)</td>
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<td>II</td>
<td>12 (17)</td>
<td>13 (31)</td>
<td>25 (23)</td>
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</tr>
<tr>
<td>III</td>
<td>6 (9)</td>
<td>5 (12)</td>
<td>11 (10)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>48 (70)</td>
<td>22 (52)</td>
<td>69 (62)</td>
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<tr>
<td>Free (&gt; 5 mm)</td>
<td>19 (33)</td>
<td>15 (36)</td>
<td>34 (34)</td>
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</tr>
<tr>
<td>Not Free (&lt; 5 mm)</td>
<td>39 (67)</td>
<td>27 (64)</td>
<td>66 (66)</td>
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<tr>
<td><strong>Extra Nodal Spread</strong></td>
<td></td>
<td></td>
<td></td>
<td>p=0.032*</td>
</tr>
<tr>
<td>Yes</td>
<td>21 (37)</td>
<td>7 (17)</td>
<td>28 (47)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>37 (64)</td>
<td>35 (83)</td>
<td>32 (53)</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>58 (100)</td>
<td>42 (100)</td>
<td>100 (100)</td>
<td></td>
</tr>
</tbody>
</table>
**Clonogenic radiation sensitivity Grenman assay**

Clonogenic radiation sensitivity Grenman assays were performed as described previously[31], [83]. Briefly, the cells were harvested with trypsin-EDTA, counted, and suspended in DMEM medium containing 10% fetal bovine serum, and plated out in 96 wells plates at 3 different amounts of cells per well. Various plates were irradiated with one single dose each of either 2 Gy, 4 Gy or 6 Gy at 0.66 Gy/min using an IBL 637 Cesium-137 γ source (CIS Biointernational, Gif-sur-Yvette, France). 14 days after irradiation the amount of positive wells per plate was determined; wells that contained at least one colony with more than 32 cells were considered positive. The plating efficiency (PE) was calculated using the formula: 

\[
PE = -\ln(\text{negative wells/total wells})/\text{cells per well}.
\]

Survival was calculated by dividing the PE after irradiation by the PE of the non-irradiated plates. The data are based on the average of 3 independent experiments.

**Western blot**

Cell lysates were separated on polyacrylamide gels and blotted onto nitrocellulose membranes using standard protocols. Blots were incubated with primary antibodies, for FADD (A66-2, BD Biosciences) or beta-actin (SC-81178, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4ºC overnight. Immunostaining was amplified by incubation with HRP-conjugated antibodies and chemiluminescence was visualized with ECL (Pierce, Rockford, USA).

For subcellular fractions the cells were suspended in a harvest buffer (10mM HEPES pH 7.9, 50mM NaCl, 0.5M sucrose, 0.1mM EDTA, 0.5% Triton X-100, freshly added HALT proteinase inhibitor cocktail (Pierce) and incubate on ice for 5 minutes. Nuclear fraction was pelleted at 1000 rpm at 4 ºC for 10 minutes. The pellet was washed and finally re-suspended in nuclear lysis buffer (10mM HEPES pH 7.9, 500mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 0.1% NP-40 and freshly added HALT protease inhibitor cocktail). The supernatant was stored as the cytoplasmic fraction. To verify whether fractionation was performed appropriate, all fractions were immunostained for PARP as control for nuclear fractionation and GAPDH as control for cytoplasmic fractionation[148].

**Immuno-fluorescence for FADD**

Cells were cultured on sterilized poly-L-lysine coated glass slides in a 6 wells plate
Clonogenic radiation sensitivity Grenman assays were performed as described previously [31], [83]. Briefly, the cells were harvested with trypsin-EDTA, counted, and suspended in DMEM medium containing 10% fetal bovine serum, and plated out in 96 wells plates at 3 different amounts of cells per well. Various plates were irradiated with one single dose each of either 2 Gy, 4 Gy or 6 Gy at 0.66 Gy/min using an IBL 637 Cesium-137 γ source (CIS Biointernational, Gif-sur-Yvette, France). 14 days after irradiation the amount of positive wells per plate was determined; wells that contained at least one colony with more than 32 cells were considered positive. The plating efficiency (PE) was calculated using the formula:

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**Immuno-fluorescence for FADD**

Cells were cultured on sterilized poly-l-lysine coated glass slides in a 6 wells plate.

---

**Table 5.2 Univariate and multivariate Cox regression analysis**

<table>
<thead>
<tr>
<th>Cox regression</th>
<th>Univariate</th>
<th>N (%)</th>
<th>Hazard Ratio for LC</th>
<th>Hazard Ratio for OS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HR  (95% CI; p-value)</td>
<td>HR  (95% CI; p-value)</td>
</tr>
<tr>
<td>pFADD</td>
<td>High</td>
<td>42 (42)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>58 (58)</td>
<td>5.3 (1.6 - 18.0; p=0.007)*</td>
<td>1.8 (1.1 – 3.1; p=0.013)*</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>42 (42)</td>
<td>1</td>
<td>1.4 (0.9 – 2.4; p=0.139)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>58 (58)</td>
<td>0.7 (0.3 – 1.6; p=0.411)</td>
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</tr>
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<td>T-stage</td>
<td>T1-T2</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>T3-T4</td>
<td>60 (60)</td>
<td>1.0 (0.4 - 2.4; p=0.979)</td>
<td>1.3 (0.8 - 2.1; p=0.261)</td>
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<td>40 (40)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>N+</td>
<td>60 (60)</td>
<td>2.2 (0.8 – 5.6; p=0.099)</td>
<td>2.6 (1.5 – 4.4; p=0.000)*</td>
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<td>1</td>
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<td>1.2 (0.5 - 3.1; p=0.591)</td>
<td>1.1 (0.7 - 1.8; p=0.701)</td>
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<td>Extra Nodal Spread</td>
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<td>1</td>
<td>1</td>
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<tr>
<td></td>
<td>Yes</td>
<td>28 (28)</td>
<td>3.8 (1.6 - 8.9; p=0.002)*</td>
<td>3.5 (2.1 – 6.0; p=0.000)*</td>
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</tr>
<tr>
<td><strong>Multivariate</strong></td>
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<td></td>
<td>HR  (95% CI; p-value)</td>
<td>HR  (95% CI; p-value)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pFADD</td>
<td>Positive</td>
<td>42 (38)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
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<td>4.2 (1.2 – 14.7; p=0.021)*</td>
<td>1.7 (1.0 – 2.8; p=0.061)</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>28 (28)</td>
<td>2.9 (1.2 - 6.9; p=0.017)*</td>
<td>1.9 (1.1 – 3.6; p=0.032)*</td>
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<tr>
<td>N-stage</td>
<td>N0</td>
<td>40 (40)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>N+</td>
<td>60 (60)</td>
<td>2.3 (1.2 – 4.1; p=0.008)*</td>
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</table>

* significant (p<0.05)
Figure 5.1 Kaplan - Meier analysis for Local Control. A. Comparing cases with high pFADD expression and low pFADD expression. B. Comparing cases with and without Extra Nodal Spread (ENS). P-values, Hazard Ratios (HR) and 95% confidence intervals (95%CI) that are displayed in the graphs are calculated by Cox regression analyses.
for 24 hours before fixation in 3.7% formaldehyde in PBS. Fixed cells were incubated with the 1:100 FADD antibody (A66-2, BD Biosciences) overnight, and then with the secondary antibody (A-21428 GaR-Alexa555, Invitrogen) for an hour at 4 °C. Afterwards the cells were treated with a solution of 10ug/ml DAPI for DNA content staining.

Statistical Analyses

Statistical analysis was carried out using the SPSS 14.0.0 software package. Associations between different markers and between markers and clinico-pathological characteristics where tested using the $\chi^2$ test. The endpoint used in

Figure 5.2 Figure 2 A Immunohistochemical staining for pFADD on HNSCC shows a primarily nuclear staining B. Immunohistochemical staining of the normal epithelium of the head and neck area shows that pFADD is highly expressed in the basal layer. C. The expression of pFADD in HNSCC is higher in cells that are in the mitotic phase of the cell cycle.
this study was local control (LC), defined as failure at the primary site only. The
time to local recurrence (LR) was calculated from the time of surgery. For the
univariate analysis, Kaplan-Meier curves were produced and compared with the
log rank test. For the multivariate analysis, a Cox regression analysis was used.
The categorized covariates that showed a trend towards statistical significance (p
< 0.10) in the univariate analysis were entered into a back-step multivariate Cox
regression analysis. Eventually, p-values < 0.05 were considered significant.

Results

*Increased expression of pFADD associates with local control in OSCC*

We performed immunohistochemical staining for pFADD on tissue specimens of
100 oral squamous cell carcinoma patients. In 42 patients (42%) high expression
of pFADD (i.e. positive staining in more than 50% of the tumor cells) was
found. The expression of pFADD was heterogeneous throughout the tumor.
Statistical analysis of pFADD expression in combination with clinico-pathological
characteristics revealed a significant negative association with extra nodal spread
(ENS) (p=0.032). No other significant associations were found (Table 5.1).

To determine whether pFADD expression was associated with LC, we performed
univariate analyses. This analysis revealed low pFADD as the strongest prognostic
factor for worse LC (HR:5.3 95%CI:1.6-18.0 p=0.007) (Table 5.2, illustrated in Figure
5.1A). Univariate analysis also revealed an association between the presence of
extra-nodal spread (ENS) and worse LC (HR: 3.8 95%CI:1.6-8.9; p=0.000) (Table
5.2, illustrated in Figure 5.1B). Multivariate analysis revealed that pFADD and ENS
were both independent significant factors for LC (HR:4.2 95%CI:1.2 - 14.7 and
HR:2.9 95%CI:1.2 - 6.9 resp.) (Table 5.2).

Univariate analysis revealed that low pFADD expression was significantly
associated with decreased overall survival (OS) (HR:1.8 95%CI:1.1-3.1 p=0.013)
(Table 5.2). Univariate analysis also revealed an association between OS and ENS
(HR: 2.6 95%CI:1.4-4.7; p=0.002) and OS and N-stage (HR: 2.6 95%CI:1.5-4.4;
p=0.000) (Table 5.2). Multivariate analysis for OS revealed that N-stage (HR: 2.3;
95%CI:1.2 – 4.1; p=0.008) and ENS (HR: 1.9; 95%CI: 1.1 – 3.6; p=0.032) were both
independent prognostic factors, while pFADD (HR:1.7; 95%CI:1.0 – 2.8; p=0.061)
showed borderline significance (Table 5.2).
Figure 5.3 FACS analysis of HNSCC cell lines immuno-stained for pFADD with DAPI staining for Cell Cycle distribution, with or without 24h treatment with 50nM taxol. Q1 represents the pFADD high/ G1 fraction, Q2 the pFADD high/G2 fraction, Q3 the pFADD low/ G2 fraction and Q4 the pFADD low/G1 fraction. Percentages of the cells per quadrant are displayed in the corner of the images, or can be found in table 5.3. A. U2 without taxol. B. U2 with taxol. C. U22b without taxol. D. U22b with taxol.

Table 5.3 The percentages per quartile as shown in Figure 5.3.

<table>
<thead>
<tr>
<th>Quadrant</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q1+Q2</th>
<th>Q2+Q3</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2 untreated (A)</td>
<td>4.5%</td>
<td>5.9%</td>
<td>31.9%</td>
<td>57.6%</td>
<td>10.4%</td>
<td>37.8%</td>
</tr>
<tr>
<td>U2 arrested (B)</td>
<td>1.2%</td>
<td>32.3%</td>
<td>48.3%</td>
<td>18.2%</td>
<td>33.5%</td>
<td>80.6%</td>
</tr>
<tr>
<td>U22b untreated (C)</td>
<td>8.2%</td>
<td>3.7%</td>
<td>29.5%</td>
<td>58.6%</td>
<td>11.9%</td>
<td>33.2%</td>
</tr>
<tr>
<td>U22b arrested (D)</td>
<td>0.4%</td>
<td>63.6%</td>
<td>30.8%</td>
<td>5.1%</td>
<td>64.0%</td>
<td>94.4%</td>
</tr>
</tbody>
</table>
Figure 5.4 A. Western blot for FADD on HEK293 stably transfected with either an empty vector (EV) plasmid, or an inducible plasmid (pIND) containing the gene for non-phospho FADD-S194A or phospho-mimicking FADD-S194E. Transfectants were either unstimulated (-) or stimulated with 5 µM Ponesterone for a 24h (+). The A66-2 FADD antibody that was used detects both phosphorylated and non-phosphorylated FADD as well as endogenous FADD expression. B. Immunofluorescence for FADD with a nuclear staining using DAPI performed of cells stimulated with PonA for 24h.
pFADD is mainly located in the nucleus and expression is increased during the G2/M phase of the cell cycle.

The outcome of our clinical data showed worse LC when pFADD expression was low suggesting that high pFADD expression is associated with high radiosensitivity. Because pFADD was reported to be most highly expressed during the G2M phase [48], [50], [149] and because cells in the G2/M phase are most sensitive to radiotherapy[150], [151], we tested expression levels of pFADD at G2/M phase in OSCC and HNSCC cell lines.

Using immunohistochemical staining, pFADD was located mainly in the nucleus of
both HNSCC cells (Figure 5.2A) and the basal layer of the normal epithelium of the head and neck area representing the proliferative fraction of the epithelium layer (Figure 5.2B). In addition, HNSCC cells in the metaphase of the cell cycle showed a higher expression of pFADD than those in the interphase (Figure 5.2C).

To determine whether cell cycle arrest at the metaphase increased the level of pFADD in HNSCC cell lines, 2 different HNSCC cell lines (UMSCC2 and UMSCC22b) with a high FADD expression were treated with 50 nm of Taxol for 16 hours. These cells were immunostained for pFADD, and simultaneously stained with DAPI to determine expression in different phases of the cell cycle. FACS analysis revealed that taxol treatment of both HNSCC cell lines resulted in a significant cell cycle arrest at G2/M and a concomitant increase of pFADD expression (Figure 5.3B&D) when compared to untreated cells (Figure 5.3A&C).

**Expression of phospho-mimicking FADD affects radio-sensitivity in vitro.**

In order to investigate the direct effect of pFADD expression on the response to radiation, we generated HEK293 transfectants that only expressed a phospho-mimicking FADD full-length isoform (FADD-S194E), or non-phospho-FADD full-length isoform (FADD-S194A) upon treatment with 5uM Ponesterone A for 24 hours. As illustrated in Figure 5.4, the Ponesterone A treated HEK293/FADD-S194A and HEK293/FADD-S194E transfectants showed an increased expression...
of the FADD protein, whereas the expression in non-treated transfectants showed very low (endogenous) expression only on western blot (Figure 5.4A) and immunofluorescence (Figure 5.4B). In the FADD-S194E overexpressing cells a double band was detected as described previously for pFADD[48], [49], [152].

To investigate whether pFADD expression conferred a higher sensitivity to irradiation, we used the in vitro Grenman survival assay. Indeed, a significantly higher radio-sensitivity was observed in the Ponasterone A treated HEK293/FADD-S194E transfectants compared to untreated cells and the non-phospho-FADD-S194A mutant overexpressing cell line (Figure 5.5). Interestingly, treated and untreated FADD-S194A transfectants both showed the same radiosensitivity as empty vector transfected HEK293 cells. These findings demonstrated that not the non-phosphorylated but only the FADD isoform phosphorylated on Ser194 causes increased radiosensitivity which is in good agreement with the observed association between high pFADD expression and better LC.

*Phospho-mimicking-FADD overexpression does not influence the cell cycle distribution*

To determine whether the effect of expression of the phospho-mimicking FADD-
S194E mutant on *in vitro* radiosensitivity resulted from cell cycle arrest, we investigated cell cycle distribution in the cells expressing the phospho-mimicking FADD mutant FADD-S194E using FACS analysis (Figure 5.6). This analysis revealed that Ponesterone A treated HEK293/FADD-S194E cells showed similar cell cycle distribution as compared to untreated HEK293/FADD-S194E cells. These data indicated that increased expression of the FADD-S194E is not sufficient to influence the cell cycle distribution to explain the *in vitro* effect on radiosensitivity.

We performed a western blot analysis on the nuclear and cytoplasmic fractions of Ponesterone A treated and untreated transfectants with FADD-S194A, FADD-S194E and empty vector as control. Appropriate fractionation was verified by immunostaining for PARP (as control for the nuclear fractions) and GAPDH (as control for cytoplasmic fractions) (Figure 5.7). This experiment revealed that upon treatment with Ponesterone A in both transfectants expressing the non-phospho FADD-S194A as well as (although at low levels) the phospho-mimicking FADD-S194E mutant, FADD is present both in the cytoplasmic fraction and in the nuclear fraction. The fractionation experiment showed that the phospho-mimicking FADD-S194E protein is also present in the nuclear fraction.

**Discussion**

Amplification of the 11q13 amplicon is frequently found in HNSCC[118], [128], [153]. Using comprehensive array-CGH and quantitative real-time RT-PCR analysis of the 13 genes located in the 11q13 region commonly amplified in HNSCC and breast cancer, we showed that FADD was the most amplified and highest overexpressed gene[38]. Recently, we showed in a well-defined cohort of T1-2 glottic carcinomas treated with definitive radiotherapy that high expression of pFADD was associated with better local control and overall survival[117]. In the current study, we showed that high expression of phosphorylated FADD was observed in 42% of locally advanced OSCC treated with surgery and postoperative radiotherapy, and was also associated with better local control and overall survival, which corresponded well with our previous findings in laryngeal carcinoma[117]. Based on these clinical data, we hypothesized that the better local control could be explained by an increased radiosensitivity of tumor cells with increased expression of pFADD. To test this hypothesis we determined changes in the *in vitro* radiosensitivity of HEK293 cells overexpressing the phospho-mimicking FADD-S194E mutant upon transcriptional stimulation. Overexpressing HEK293/FADD-S194E cells showed significantly decreased cell survival after irradiation.
Interestingly, when cells expressing the non-phospho FADD-S194A mutant were used, no effect on cell survival was observed. This is in good agreement with the immunostaining results on the same tissue specimens of 100 OSCC patients using an anti-FADD monoclonal antibody (clone A66-2) that detects expression of all FADD isoforms. High expression of FADD was observed in 49% of OSCC[146], but statistical analysis revealed no association between high FADD expression and LC (HR: 1.2; 95%CI: 0.5-2.7; p=0.6) (data not shown). From these findings, we concluded that not the expression levels of FADD but the phosphorylation status of FADD is responsible for the observed effect on radiosensitivity.

With immunostaining, a very high expression of pFADD was observed in those cells that showed clear mitotic patterns (Figure 5.4B), confirming the observation that pFADD expression is higher in the G2/M phase of the cell cycle. To investigate the effect of the cell cycle distribution on the expression of pFADD in HNSCC we treated 2 HNSCC cancer cells lines (UMSCC2 and UMSCC22b) with taxol to induce a G2/M arrest. By using 50 nM taxol for 24h, both cell lines were fully arrested in the G2/M phase and the arrested cells showed a significant shift to high pFADD expression at G2/M when compared to the untreated cells. Also in human cell lines other than HNSCC (BJAB, HeLa, MDA-MB-231 and MCF7) arresting cell cycle at the G2/M phase showed high pFADD at G2/M and low at G1/S[50], [51].

The establishment of stable cell lines continuously overexpressing full-length FADD was not successful. This was most probably due to the fact that increased FADD expression causes apoptosis[154] as after transfection no transfectants were found expressing the FADD transgene (data not shown). This is in good agreement with the literature as most other studies that generated FADD overexpressing cell lines used a truncated isoform (C-FADD) not inducing apoptosis since it lacks the DED domain[49], [50]. Since we cannot exclude that the DED domain is involved in the observed radioresponse of pFADD overexpressing cells, we generated stable cell lines with a full-length FADD under control of the Ecdysone promoter that is only induced upon treatment with Ponesterone A, an insect hormone which is not present in eukaryote cells. For this study we selected transfectants that only showed transgene expression upon Ponesterone A treatment for 24 hrs.

Since the G2/M phase of the cell cycle is the most radiosensitive phase[150], [151], we investigated whether the effect of phospho-mimicking FADD-S194E mutant on the radiosensitivity of Hek293 cells was due to an increased fraction of the cells being in the G2/M phase of the cell cycle. Our cell cycle experiments showed that
cells overexpressing FADD-S194E did not have a different cell cycle distribution compared to those cells with no FADD-expression. Similar observations were reported in BJAB, HeLa, MDA-MB-231 and MCF7 cell lines transduced with the truncated (lacking DED domain) C-FADD-S194E mutant[49], [155]. These results indicate that the increased sensitivity to radiation that is associated with pFADD expression, is not due to a cell cycle effect.

In this study, immunostaining of OSCC showed that pFADD was predominantly localized in the nucleus. In addition, we showed that pFADD expression increased when the cells were arrested in the G2/M phase of the cell cycle, which corresponds well with earlier studies[49], [51], [132], [155]. It is generally accepted that transition to and localization in the nucleus is important for FADD to be active on cell cycle[48], [49], [51], [132], [155] and phosphorylation of FADD is essential for its nuclear localization[48], [49], [51], [132], [155]. To verify that FADD-S194E is indeed translocated into the nucleus, we performed subcellular fractionation analysis. In Ponesterone A treated HEK293/FADD-S194E cells, cellular fractionation revealed a predominant cytoplasmic rather than nuclear localization, whereas the non-phospho-FADD-S194A mutant was equally present in the cytoplasm and in the nucleus. From this observation we concluded that the translocation from the cytoplasm to the nucleus is easier for non-phosphorylated FADD then for phospho-mimicking FADD under the circumstances used in this study. So far the mechanism by which FADD is translocated to the nucleus remains unclear. In previous studies it was reported that Ser194 phosphorylation plays an important role in both the nuclear import and export[49], [132], [149], [155]. For instance, Screaton et al. showed that the localization of FADD was primarily nuclear in most cell lines, yet the non-phospho-FADD-S194A mutant caused a more homogeneous distribution between cytoplasm and nucleus[149]. It was also shown that the export from the nucleus to the cytoplasm was more efficient for the FADD-S194A mutant. These data using truncated FADD isoforms are in very good agreement with our observations using the full-length FADD isoform. One explanation for the fact that FADD-S194E in our study is detected at very low levels in the nucleus only, is that for both the import to the nucleus and the export to the cytoplasm, FADD needs to be in the un-phosphorylated state. Upon phosphorylation in the nucleus, pFADD would be kept in the nuclear FADD pool, whereas de-phosphorylation would release FADD into the cytoplasm. Another argument for this mechanism is that casein kinase I alpha (CK1α), one of the prominent enzymes that phosphorylate FADD specifically at Ser194, is co-localized
with pFADD on the mitotic spindles during the early anaphase of the cell cycle [15], indicating that the phosphorylation of FADD is performed in the nucleus. To further investigate the role of nuclear pFADD on cell cycle regulation and radiosensitivity, FADD-mutants containing nuclear import or export signals could be used as previously reported[149]. Although the mechanism is not yet completely understood presently, our data showed that not the non-phospho-FADD but the FADD isoform phosphorylated on Ser194 is involved in radiosensitivity.

In conclusion, we showed that high expression of pFADD in OSCC treated with primary surgery and postoperative radiotherapy is associated with better local control. Our in vitro experiments provided more direct evidence that neither the FADD expression levels, nor an effect on cell cycle distribution, but the phosphorylation status of FADD results in an increased sensitivity to radiation in Hek293 cells. Further research is needed to unravel the biological mechanism by which FADD confers radiosensitivity to HNSCC cells.