Chapter 3

FADD expression is associated with regional and distant metastasis in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Background

The Fas Associated Death Domain (FADD) gene is often overexpressed in squamous cell carcinoma of the head and neck (HNSCC) and is considered to be a driver gene in DNA amplification of the chromosomal 11q13.3 region. Amplification of 11q13.3 is associated with increased metastasis in HNSCC and breast cancer. The aim of this study was to correlate FADD expression in advanced stage HNSCC with clinico-pathological features and clinical outcome.

Patients and Methods

Tumor tissues of 177 HNSCC patients uniformly treated with primary surgery and postoperative radiotherapy were collected. FADD expression was assessed on pre-treatment tumor biopsies using immunohistochemistry.

Results

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 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.

Conclusion

Our data show that an increase in FADD expression is associated with a higher incidence of lymph node metastasis at presentation and with shorter DMFI when lymph node metastases are present. High FADD expression in the primary tumor could be a useful marker to select patients for systemic treatment strategies that reduce the risk for distant metastases.
Introduction

Head and neck squamous cell carcinoma (HNSCC) is the 6th most common type of cancer worldwide[1], [2], with an incidence of approximately 9.2 per 100,000 people in 2008[4]. The most important risk factors for this type of cancer are smoking, alcohol consumption and infection with the human papillomavirus (HPV)[5], [6].

Although outcome of locally advanced HNSCC has been improved significantly with combined modality approaches, still about half of the patients develop loco-regional recurrences and distant metastases[2], [3], [109]. Accurate estimation of outcome in HNSCC based on clinical prognostic factors, such as TNM-stage, remains difficult. Therefore, there is need for a more precise prediction of outcome in order to adjust treatment modalities to the risk of either loco-regional failure or distant metastasis. During the last decade, increasing data became available on molecular and genetic changes associated with malignancy and tumor progression. Some of these genetic changes are specifically identified in HNSCC[38], [110], [111] and may be good candidates for predicting clinical outcome in HNSCC patients.

The most commonly amplified region in HNSCC is chromosome 11q13.3[37], [110]. Previous studies have shown that amplification of 11q13.3 correlates with worse clinical outcome, both in HNSCC[112] and in breast cancer[113]. In HNSCC, we previously showed that within the commonly amplified 11q13.3 region, a cluster of 13 genes is most frequently co-amplified, including 9 genes (FADD, PPFIA1, TPCN2, CCND1, FLJ42258, ORAOV1, ANO1, FGF19 and CTTN) that are overexpressed when amplified. From the genes within the core of the amplicon, the expression of the gene coding for the Fas Associated Death Domain (FADD) correlates best with 11q13.3 amplification status[38].

Originally, FADD was reported in the extrinsic apoptosis pathway to act as an adaptor linking the death receptors to caspase-8 and passing the extracellular apoptosis signals onto the intracellular caspases, eventually resulting in apoptosis[42]–[44]. However, recently other functions have also been attributed to FADD, such as enhancing in vitro invasion, inhibiting necrosis in epithelial cells and regulating cell proliferation in both epithelial and lymphoid cells (see for review[43], [114]–[116]).

The current study was designed to investigate the role of FADD expression in the
metastatic potential in terms of regional and distant metastases in a retrospective cohort study consisting of HNSCC patients uniformly treated with surgery and postoperative radiotherapy.

Methods

Patients and tissues

The study cohort consists of consecutive patients diagnosed with HNSCC, uniformly treated with primary surgery and postoperative radiotherapy at the University Medical Center Groningen between 1993 and 2003. Clinical and histopathological data of all patients were collected (n=198) as described previously[31]. The follow-up was at least 3 years.

Formalin-fixed paraffin-embedded surgically resected tissues of the primary tumors were collected and revised by an experienced pathologist. In the current study we included 177 patients for whom the immunohistochemical staining for FADD was available. The study cohort mainly consisted of male patients (64%) with a median age of 60 years (range 24–90), with predominantly loco-regional advanced stages. Based on histopathological examination of the surgical specimen, 75% of the patients had advanced primary tumors (T3-T4) and 64% had lymph node metastasis (N+) with 84% of the patients having stage III or IV disease.

All 177 patients underwent surgery of the primary tumor and in 151 cases this was combined with a neck dissection. All patients were treated with postoperative radiotherapy based on pathological risk factors, such as positive surgical margins (69%), lymph node metastases with extranodal spread (36%) and/or other adverse prognostic factors such as advanced T-stage, multiple lymph node metastases and/ or perineural growth. None of the patients received postoperative chemoradiation at that time.

Immunohistochemistry

Immunohistochemical staining was performed as described previously[38], [117]. Briefly, paraffin-embedded, formalin-fixed, 3 µm thick sections of tumor tissue were deparaffinized and rehydrated in a gradient series of alcohol. Antigen retrieval was performed on all specimens by incubating overnight at 80°C in 0.1 M Tris/HCl (pH 9.0). Subsequently, the endogenous peroxidase was blocked in a 0.3% H₂O₂ solution. The slides were incubated with mouse – anti FADD monoclonal antibody
clone A66-2 (BD Pharmingen, San Jose, CA, USA) 1:100 diluted in PBS for one hour. This was followed by Horseradish Peroxidase (HRP) conjugated Rabbit anti Mouse (RaM<sub>Po</sub>) immunoglobulin G (IgG) (1:100). Finally the slides were incubated with horseradish peroxidase conjugated goat anti rabbit (GaR<sub>Po</sub>) IgG (1:100) for one hour. The slides were developed with 3,3′-di-aminobenzidine (DAB) chromogen solution (Dako, Glostrup, Denmark) and counterstained using haematoxylin. All cases were evaluated independently by two observers without prior knowledge of clinical data. In case of discrepancies between the observers, cases were re-evaluated with an experienced pathologist until consensus was reached.

Cytoplasmic staining intensity was semi-quantitatively scored as: negative (0); weakly positive (+); positive (++) or strongly positive (+++) staining as previously described<sup>9</sup>. For statistical purposes the cases with 0 or + were classified as low FADD, whereas the ++ and +++ cases were categorized as high FADD (Figure 3.1).

Statistical analysis

Statistical analysis was carried out using the SPSS 14.0.0 software package (SPSS Inc., Chicago, IL, USA). Associations between FADD expression and clinicopathological characteristics were tested on statistical significance using the Chi square-test. The primary endpoint used in this study was distant metastasis.

Figure 3.1 FADD staining for a negative (A, B, C) and a strong positive (D, E, F) case.
Table 3.1 A. Clinico-pathological characteristics of the patient series, stratified by FADD expression
B. Cross table between FADD staining and Extra Nodal Spread, calculated only for the lymph node positive patients.

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free interval (DMFI), which was defined as the time from surgery until the first distant metastasis in the case of an event or from the time from surgery until the death or last follow-up date in the case of no event. For the univariate and multivariate analysis of clinical outcome, a Cox regression analysis was used. The categorized covariates with a p-value of 0.10 or smaller in the univariate analysis were put into a back-step multivariate Cox regression analysis. P-values 0.05 were considered significant. Kaplan – Meier survival curves for DMFI were created to illustrate the differences.

Results

*FADD expression is associated with N-stage*

Immunohistochemical cytoplasmic staining for FADD revealed 99 (56%) low FADD
and 78 (44%) high FADD cases. In order to assess whether FADD expression was associated with clinico-pathological characteristics (age, gender, T-stage, N-stage, stage and surgical margin status) we performed cross-table analyses (Table 3.1A). Analysis of the association between FADD expression and extranodal spread (ENS) was only performed on the subpopulation of patients who underwent a neck dissection and were diagnosed with a lymph node metastasis positive neck (n=107) (Table 3.1B). The expression of FADD was significantly associated with the presence of lymph node metastasis (N0 vs. N+; \( p=0.001 \)), however not with any of the other characteristics tested.

**FADD expression is associated with increased risk for distant metastasis**

In order to assess the association between FADD expression and clinical outcome we performed univariate Cox regression analyses for the distant metastasis free interval (DMFI). These analyses revealed that FADD expression was significantly
associated with a shorter DMFI (HR: 3.3, 95%CI: 1.3-8.1, \( p=0.008 \)) (Table 3.2; Figure 3.2A). We also analyzed the association between clinico-pathological characteristics and clinical outcome. These analyses revealed that, besides FADD also N-stage was significantly associated with shorter DMFI (Table 3.2; Figure 3.2B). In a multivariate analysis, N-stage was the only significant factor associated with DMFI (HR: 14.5, 95%CI: 1.9-108.5, \( p=0.009 \)). A trend towards a shorter DMFI was found with high FADD expression (HR: 2.3, 95%CI: 0.96-5.7, \( p=0.062 \)) (Table 3.3).

*FADD expression predicts shorter distant metastasis free interval in N+ patients*

To investigate the effect of the FADD expression within the group of cases with positive lymph nodes, we divided the study cohort in 3 groups: patients with N0, patients with N+/FADD- and patients with N+/FADD+ tumors. In multivariate analysis, the DMFI was significantly shorter for FADD+ compared to the FADD- cases within the N+ group (HR:2.6, 95%CI:1.0-6.7, \( p=0.046 \)) (Table 3.4, Figure 3.2C). When analyzing the subgroup of N+...
tumors (n=107) in a multivariate analysis, besides FADD also ENS (HR: 2.8, 95%CI: 1.1-7.2, \( p=0.034 \)) was an independent predictor for a shorter DMFI (data not shown).

Discussion

The 11q13.3 region, in which the FADD gene is located, is amplified in ~ 36% of the cases of HNSCC\[118\] and FADD expression is highly associated with the presence of this 11q13.3 amplification\[38\]. We hypothesized that overexpression of FADD is beneficial for HNSCC tumor cells and in consequence may drive this amplification\[38\].

In the current study we showed that in our study cohort of mainly advanced stage HNSCC treated with primary surgery and postoperative radiotherapy, high FADD expression was detected in 78 of the 177 cases (44%), and that this high expression was associated with a higher incidence of lymph node metastasis. Moreover, high FADD expression was associated with a shorter distant metastasis free interval (DMFI), with a 3.3 times higher risk for developing a distant metastasis.

We previously performed FADD expression studies on an independent, and heterogeneously treated group of mainly advanced laryngeal squamous carcinoma patients collected from multiple centers. In that study we found high FADD expression in 62/140 (44%) of the cases, and a significant association with worse survival\[119\]. The current study was performed on a consecutively and homogeneously treated group of patients from a single center, with a longer follow-up of at least three years. The comparable percentage of high FADD expression in these two different populations of HNSCC shows that high FADD expression is a frequent event in advanced HNSCC. The lower incidence of 26% overall positivity in the recent study of Rasamny et al.\[120\], might be due to the heterogeneous group of HNSCC from nearly all sub-localizations and generally less advanced tumors, (55% N0 versus 39% in our study). A Japanese study of 60 early-stage tongue SCC reported FADD amplification in 44%, FADD expression was only assessed quantitatively and positive in 33%\[25\]. In both studies FADD was associated with N status and shorter disease-specific survival.

Because FADD has been associated with lymph node metastasis, and shorter survival, not only in HNSCC\[120\], \[121\], but also in breast cancer\[122\], the current study was set up to further analyze the role of FADD in the metastatic potential in a
clinical series of HNSCC. Both N status and distant metastasis free survival (DMFI), which directly reflects metastatic progression of the disease, were associated with FADD expression.

FADD was originally described as an adapter molecule in the DISC to mediate apoptose through activation of caspase-8[43], [44]. However, more recent studies showed a much more complex and largely unresolved role, FADD being involved in apoptosis, embryonic development, cell survival, proliferation, tumor progression, TLR-signaling, inflammation, necrosis and autophagy, partially by interacting with other molecules such as Atg5 and RIPK1/3 complexes (see for review[43], [114], [116], [123]). From these studies it emerged that FADD expression can lead to apoptosis but also to inhibition of apoptosis or necrosis[115], [124]–[126]. Moreover, the balance between phosphorylated and non-phosphorylated FADD and in consequence, nuclear versus cytoplasmic FADD may be closely associated with carcinogenesis[51]. On the other hand, the nuclear localisation of FADD has also been suggested for storage in resting cells and enabling immediate redistribution to the cytoplasm upon CD95 activation[127]. Of note, we used the A66-2 antibody directed against FADD irrespective of its phosphorylation status, in particular since it has been suggested that cancer cells express high levels of unphosphorylated FADD in comparison to their normal counterparts[38]. We also investigated with the expression of phosphorylated FADD using a specific antibody[38] but expression was not associated with a higher incidence of lymph node metastasis (data not shown). Therefore, the underlying mechanism how FADD leads to an increased metastatic potential as shown in the current study, remains to be elucidated.

In summary, our data show that an increase in FADD expression is associated with a higher incidence of lymph node metastases at presentation and is associated with shorter DMFI when lymph node metastases are present. Therefore high FADD expression in the primary tumor could be a useful marker to select patients for systemic treatment strategies that reduce the risk for distant metastases.