Proteomic Profiling Identifies PTK2/FAK as a Driver of Radioresistance in HPV-negative Head and Neck Cancer

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Abstract

**Purpose:** Head and neck squamous cell carcinoma (HNSCC) is commonly treated with radiotherapy, and local failure after treatment remains the major cause of disease-related mortality. To date, human papillomavirus (HPV) is the only known clinically validated, targetable biomarkers of response to radiation in HNSCC.

**Experimental Design:** We performed proteomic and transcriptomic analysis of targetable biomarkers of radioresistance in HPV-negative HNSCC cell lines in vitro, and tested whether pharmacologic blockade of candidate biomarkers sensitized cells to radiotherapy. Candidate biomarkers were then investigated in several independent cohorts of patients with HNSCC.

**Results:** Increased expression of several targets was associated with radioresistance, including FGFR, ERK1, EGFR, and focal adhesion kinase (FAK), also known as PTK2. Chemical inhibition of PTK2/FAK, but not FGFR, led to significant radiosensitization with increased G₂-M arrest and potentiated DNA damage. PTK2/FAK overexpression was associated with gene amplification in HPV-negative HNSCC cell lines and clinical tumors. In two independent cohorts of patients with locally advanced HPV-negative HNSCC, PTK2/FAK amplification was highly associated with poorer disease-free survival (DFS; \( P = 0.012 \) and 0.034). PTK2/FAK mRNA expression was also associated with worse DFS (\( P = 0.03 \)). Moreover, both PTK2/FAK mRNA (\( P = 0.021 \)) and copy number (\( P = 0.063 \)) were associated with DFS in the Head and Neck Cancer subgroup of The Cancer Genome Atlas.

**Conclusions:** Proteomic analysis identified PTK2/FAK overexpression as a biomarker of radioresistance in locally advanced HNSCC, and PTK2/FAK inhibition radiosensitized HNSCC cells. Combinations of PTK2/FAK inhibition with radiotherapy merit further evaluation as a therapeutic strategy for improving local control in HPV-negative HNSCC. *Clin Cancer Res*; 1–8. ©2016 AACR.

Introduction

Head and neck squamous cell carcinoma (HNSCC) kills approximately 200,000 people a year (1), most from locoregional recurrence. One of the primary modes of therapy for this disease is radiation. Although the localized manifestation of HNSCC suggests that proper local therapy can be curative, the disease frequently exhibits intrinsic radioresistance. Indeed, disease recurs locally in up to 50% of patients after radiotherapy for locally advanced HNSCC, ultimately leading to their demise (2–4).

Given the importance of radiation in the management of HNSCC, a variety of studies have been performed to identify biomarkers of radioresistance in this disease. A notable success story in this effort is the recognition that human papillomavirus (HPV)–driven HNSCC is associated with much higher cure rates than HPV-negative disease (5). In patients with HPV-negative HNSCC, mutations in TP53 (6) and altered function of EGFR and PI3K/AKT signaling have been implicated in HNSCC radioresistance (6–9). Preclinical studies of EGFR activation in HNSCC ultimately culminated in a clinical trial investigating the use of cetuximab (an EGFR-targeted antibody) in combination with radiotherapy (4). However, in this trial, radiotherapy failed in about 50% of patients even with EGFR inhibition. Also, the most recent randomized trial investigating cetuximab showed no improvement in outcome when used in combination with chemoradiation (2). Thus, additional “targetable” biomarkers of radioresistance in HPV-negative HNSCC are needed.

To this end, we conducted a comprehensive analysis of both protein and gene expression in a large panel of HPV-negative
HNSCC cell lines. Specifically, we used reverse-phase protein array (RPPA) to analyze 177 proteins and phosphoproteins in 49 head and neck cancer cell lines to identify proteins most strongly associated with radioresistance, with similar analyses of mRNA expression. We identified and validated the importance of focal adhesion kinase (FAK/PTK2) in radioresistance, showing that chemical inhibition of its kinase function resulted in consistent radiosensitization. Notably, we further found that PTK2/FAK was associated with treatment failure after radiotherapy in several separate cohorts of patients with HPV-negative HNSCC.

Materials and Methods

Cell lines

The 49 HNSCC cell lines used for this study, generously supplied by Dr. Jeffrey Myers through The University of Texas MD Anderson Cancer Center Head and Neck cell line repository or acquired from the ATCC, are shown in Supplementary Table S1. All cell lines were cultured in DMEM containing 10% FBS, penicillin/streptomycin, glutamine, sodium pyruvate, nonessential amino acids, and vitamins. Each cell line was subjected to short tandem repeat genomic profiling for authentication. HN31 HNSCC cell lines stably expressing either scrambled short hairpin (shRNA) or shRNA specific for PTK2/FAK were generated as described previously (6).

Copy number and mRNA expression

SNPs were analyzed with SNP 6.0 or CytoScan HD Arrays (Affymetrix), and the data were analyzed with Partek (v6.6, Partek Inc.), ASCAT (v2.1), and R software as described previously (10). Expression of selected mRNAs was analyzed with Illumina HumanWG-6 V3 BeadChip human whole-genome expression arrays as described in the Supplementary Methods.

Reverse-phase protein array

Protein lysate was collected from each cell line under full-serum (10% FBS) or serum-starvation (0% FBS) conditions for 24 hours before collection, or under serum-starved conditions (24-hour serum starvation followed by 30 minutes of 10% FBS before collection) and subjected to RPPA analysis as described previously (ref. 11; Supplementary Methods). Cell lines were separated into resistant and sensitive groups by median surviving fraction at 2 Gy (SF2). Proteins and phosphoproteins that were expressed at a ≥1.5-fold difference between the two groups, with a FDR of 1%, were examined (12).

In vitro radioresponse

Clonogenic cell survival was assessed in all HNSCC cell lines as described previously (6). Cell cycle was analyzed by flow cytometry of propidium iodide–stained DNA. All experiments were done up to three times for each cell line. Additional details are found in the Supplementary Methods.

Clinical data

Patient data were collected either from patients treated at MD Anderson Cancer Center or from the Cancer Genome Atlas (TCGA) public HNSCC database. All MD Anderson patients received surgical resection followed by postoperative radiotherapy, generally to 60 Gy, from 1990 through 2003. Two separate cohorts of patients were acquired and analyzed by two independent groups at MD Anderson as part of ongoing projects. Tumor sections were stained for p16 where available, and p16-positive tumors were excluded from the study (n = 5). Records in the HNSCC cohort of TCGA database are incomplete with regard to therapy, but most patients were treated with surgery, with many receiving postoperative radiotherapy. Patients with known HPV-positive tumors and patients lacking follow-up information were excluded from the analysis. Tumor characteristics are shown in Supplementary Table S2, and other information on clinical data analysis is included in the Supplementary Methods. Univariate analysis was done with Cox regression examining tumor stage, nodal stage, site, and PTK2 expression. Variables with a $P<0.1$ were included in the multivariate model and forward stepwise Cox regression was performed. For PTK2/FAK mRNA expression, time-dependent ROC curve analysis was used to assess the performance of survival prediction by comparing the AUC between different time points from two different cohorts (MD Anderson and TCGA; ref. 13). Because most events took place within 12 months of treatment in the MD Anderson cohort, the ability of models to predict outcome at and around 12 months was assessed with permutation tests; the P values of the AUC were calculated from 1,000 permutations of the survival data. Survival curves were generated by using the method of Kaplan–Meier, with log-rank statistics used to determine significance. R software, SPSS statistical software (v20) and GraphPad Prism were used for clinical data analysis. Additional information on mRNA and copy number expression in the clinical samples is given in the Supplementary Methods.

Results

Cell line analysis identifies proteins associated with radioresistance in HNSCC

To identify novel targets for radiosensitization, we surveyed 49 HNSCC cell lines for their response to radiation and imposed a
also performed immunoblot confirmation of the increased levels of PTK2/FAK in radioresistant HNSCC cell lines seen on RPPA (Supplementary Fig. S1).

RPPA identified a number of proteins that were differentially expressed between radioresistant and sensitive HNSCC cell lines. Specifically, 4EBP1 p65, cyclin D1, IGFR, PTCH, phosphorylated Smad3, and Stat 3 were all significantly downregulated in radioresistant cell lines (Fig. 1B). However, because the primary focus of the current work was to identify radiosensitivity biomarkers that are directly targetable, we turned our attention to phosphorylated EGFR, EN1, ERK1, FAK, and FGFR1, all of which were upregulated in the radioresistant cell lines. These targets, EGFR activation is well known to be associated with radiosensitivity in HNSCC (14), providing at least partial validation for this RPPA-based approach. EN1 is a homeobox transcription factor primarily regulating neural development (15). Although further investigation of its role in cellular response to radiation may be interesting, at present no drugs directly targeting this protein are available. Thus, ERK1, FAK, and FGFR1 seem to be the most promising targetable biomarkers for radiosensitivity in HNSCC based upon RPPA.

We next compared gene expression by using mRNA array of the proteins differentially expressed between the two groups in our RPPA screen. Similar to our RPPA results, we found increased gene expression of PTK2/FAK in radioresistant compared with sensitive cell lines \( (P = 0.03; \text{Fig. 2A and B}) \). However, neither ERK1 nor FGFR1 mRNA were expressed at significantly different levels between resistant and sensitive HNSCC. From this combined analysis, PTK2/FAK emerged as a highly significant marker of radiosensitivity at both the mRNA and protein expression levels. PTK2/FAK is also interesting as a therapeutic target, as several inhibitors are currently in or have completed phase I/II trials (16).

Figure 1.
Proteomic profiling identifies markers of radiosensitivity in HPV-negative HNSCC cell lines. A, reverse-phase protein array analysis of 49 HNSCC cell lines, illustrated as unsupervised hierarchical clustering. Shown are proteins and phosphoproteins differentially expressed between radioresistant (red bars) and radiosensitive (blue bars) cell lines (FDR 1%, mean fold difference ≥ 15). B, graphical representation of proteins and phosphoproteins differentially expressed between groups.
Inhibition of PTK2/FAK leads to significant radiosensitization and potentiation of DNA damage

Next, to examine whether pharmacologic inhibition of PTK2/FAK leads to radiosensitization in HNSCC, we treated HN5 cells with radiation in combination with PF00562271, which blocks the ATP-binding domain of FAK, rendering it reversibly catalytically inactive. For comparison purposes, we also examined nintedanib, which inhibits FGFR as well as VEGF and platelet-derived growth factor. With colony formation used as our readout of response, we found that nintedanib did not result in increased sensitivity to up to 6 Gy of radiation in HN5 cells (Fig. 3A). Conversely, inhibition of PTK2/FAK reduced the surviving fraction in HN5 cells at all three doses of radiation tested (Fig. 3B), at statistically significant levels or trending toward significance (P = 0.06). Moreover, shRNA-targeting PTK2/FAK showed radiosensitization as well (Supplementary Fig. S2A and S2B). To
PTK2/FAK copy number is highly expressed in HNSCC

On the basis of these findings, we investigated the utility of PTK2/FAK as a clinically targetable biomarker of radioresistance. Because a candidate biomarker of radioresistance should be expressed at levels high enough to be routinely detectable in a malignancy of interest in addition to being potentially targetable, we assessed PTK2/FAK gene expression in a large, publicly available database of HNSCC cell lines (www.oncomine.com; ref. 17) and HNSCC tumors (TCGA Research Network; ref. 18; Supplementary Fig. S3A and S2B). Analysis of both cell lines and tumors confirmed high levels of PTK2/FAK expression in compared with other solid tumors, indicating its possible importance in HNSCC.

PTK2/FAK copy number is highly associated with mRNA and protein expression

To further investigate the nature of FAK/PTK2 overexpression in HNSCC, we measured PTK2/FAK copy numbers in our panel of HNSCC cell lines. Both PTK2/FAK gene expression and protein levels were found to be highly correlated with FAK/PTK2 copy number in those cell lines (Fig. 4A). An additional analysis of HNSCC tumors from the publically available data in TCGA (Fig. 4B) also showed that PTK2 copy number was highly associated with gene expression and protein level, with amplifications in PTK2/FAK leading to increased PTK2/FAK expression. These data suggest that PTK2/FAK copy number amplification is a mediator of its overexpression in HNSCC.

PTK2/FAK is associated treatment failure in several independent cohorts of patients with HPV-negative HNSCC

Finally, to determine whether FAK/PTK2 was associated with locoregional failure following radiation treatment, we analyzed PTK2/FAK copy number in archival tumors from two separate cohorts of patients with locally advanced HNSCC treated with surgery and postoperative radiotherapy at MD Anderson and determined its relationship to DFS (tumor characteristics in Supplementary Table S2). Copy number was determined separately for each cohort by independent investigators blinded to outcome. In the first cohort, PTK2/FAK copy number was significantly associated with DFS on univariate analysis (HR = 1.67; P = 0.012; Supplementary Table S3). Specifically, patients with FAK/PTK2 amplification had significantly worse outcomes and experienced disease recurrence significantly sooner than remaining patients. All of the patients with amplified PTK2 had experienced treatment failure by 24 months, whereas 70% of patients with either no alteration in FAK/PTK2 copy number or a deletion were disease-free at that time (Fig. 5A). This finding was confirmed in a second cohort of patients, that amplification of PTK2/FAK was a significant negative predictor of DFS (Fig. 5B). Again, this finding was significant on both univariate (HR = 1.74; P = 0.031; Supplementary Table S3) and multivariate analysis (HR = 1.53; P = 0.034; Supplementary Table S3). Combining these two cohorts to increase the statistical power of our analysis further validated that PTK2 was associated with radioresistant head and neck cancer (Fig. 5C). Furthermore, separate validation of this finding, we examined PTK2/FAK copy number in publically available data available from TCGA Research Network (tumor characteristics in Supplementary Table S4; ref. 18). As shown in Fig. 5D, PTK2/FAK trended toward association with DFS (P = 0.063) in all patients, moreover DFS was significantly different between patients whose tumors expressed amplified PTK2 FAK (median DFS, 18.9 months) compared with those with either a deletion or no change in PTK2/FAK (median DFS, 53.1 months; P = 0.05). Again, this finding was significant on multivariate analysis (HR = 1.6; P = 0.034; Supplementary Table S3). To further extend these findings, we also examined FAK/PTK2 mRNA expression in a group of 102 patients with locally advanced HNSCC treated with surgery and radiotherapy (Fig. 6A). Similar to PTK2/FAK copy number, higher levels of FAK/PTK2 mRNA expression were associated with worse DFS (P = 0.03). To validate this finding, we examined the Head and Neck Cancer TCGA cohort and again found that PTK2/FAK mRNA expression was associated with DFS (P = 0.021; Fig. 6B). Specifically, median DFS was not reached in patients with tumors expressing low levels of PTK2/FAK mRNA, compared with a median DFS of 35 months in
patients with tumors expressing high levels of PTK2/FAK mRNA ($P = 0.036$; Fig. 6C).

**Discussion**

We undertook a systematic, high-throughput search to identify biomarkers of radioresistance that can be targeted with agents currently available in clinical trials. By using comprehensive proteomic and genomic analysis, we identified several druggable kinases upregulated in HNSCC cell lines that are resistant to radiation, a finding suggesting that these proteins may be important in recurrence of HNSCC after radiation treatment as well as being novel targets for biologically driven radiosensitization. Our methods were at least partially confirmed by our identification of upregulation of EGFR, a marker of radioresistance whose inhibitor, cetuximab, is the only FDA-approved radiosensitizer in HNSCC. Although other signaling pathways had significant differences in key enzymes between sensitive and resistant cell lines, many of those identified were significantly downregulated in the resistant cells, which does not allow direct targeting for radiosensitization.

Two kinases identified as potential biomarkers for radioresistance in HNSCC were FGFR1 and FAK. Amplification of the FGFR1 gene has been associated with worse outcomes in patients with oral SCC (19). Several tyrosine kinase inhibitors targeting FGFRs are currently under evaluation and one such inhibitor, nintedanib, has been shown to prolong survival when used in combination with docetaxel in lung adenocarcinoma (20). However, in our study pharmacologic inhibition of FGFR1 did not radiosensitize HN5 HNSCC cells, nor was FGFR1 copy number associated with DFS (data not shown). Thus, although we cannot rule out FGFR inhibition as a possible therapy, we focused our attention primarily on PTK2/FAK.
showing DFS in patients from the TCGA cohort split at median PTK2/FAK expression.

In this study, we identified the importance of PTK2/FAK to in vitro radioresistance in HNSCC at both the proteomic and gene expression level. Inhibition of PTK2/FAK function led to radiosensitization in several cell lines, primarily due to G2–M arrest and unrepaired DNA damage. Most importantly, PTK2/FAK was validated as a marker of failure after radiotherapy in several cohorts of patients with HNSCC. This provides strong evidence of the clinical importance of PTK2/FAK and the significance of targeting this kinase for radiosensitization in this disease.

Although PTK2/FAK is highly expressed in HPV-negative HNSCC, the cause of PTK2/FAK overexpression is not clear. This gene is not routinely mutated in HNSCC, with mutations being seen in only 2% of HNSCC patients (TCGA Research Network; ref. 18). Although PTK2/FAK copy number gain or amplification is observed in approximately 70% of cases, some work indicates that PTK2/FAK protein expression is not driven by copy number amplification (18, 21). However, studies in breast and lung cancer have directly linked PTK2/FAK copy number with both mRNA and protein expression (22, 23). In the current study, we provide additional evidence of a direct link between PTK2/FAK amplification and expression in HPV-negative HNSCC.

Notably, the canonical role of PTK2/FAK in cancer progression has concerned metastatic spread. That is indeed logical, as its primary function is in regulation of focal adhesions and cell-to-cell interactions through integrin binding (24). However, HNSCC is far less likely to metastasize than many solid tumors, and death is primarily due to locoregional recurrence. Yet here we identify PTK2/FAK as both an in vitro and clinical marker of radioresistance that seems to be targetable to achieve radiosensitization. Indirect targeting of PTK2/FAK via β1-integrin inhibition and direct targeting via siRNA can achieve radiosensitization, possibly via alteration of JNK kinase signaling (25, 26). Selective targeting of PTK2/FAK in endothelial cells can also radiosensitize tumors in preclinical lung carcinoma and melanoma models (27). At least one study, however, has reported that knockout of PTK2/FAK in SCC cells and in a preclinical xenograft model leads to radioresistance (28); the reason for these discrepant findings is not known. Interestingly, the preclinical model for the latter study used SCC-expressing wild-type p53, whereas previous studies have examined cells expressing primarily mutant p53, which represent the vast majority of both HNSCC cell lines and tumors (29, 30). PTK2/FAK and p53 are known to interact on several levels, with wild-type p53 functioning as a transcriptional repressor of PTK2/FAK (31) as well as directly binding PTK2/FAK protein (32, 33). Small-molecule disruption of this binding seems to have some antitumor effect via reactivation of the apoptotic function of p53 (34). Also, in at least one study TP53 mutation was correlated strongly with PTK2/FAK overexpression in breast cancer (35). Thus, the conflicting results of PTK2/FAK inhibition on radiosensitization may be partially p53-driven, as a group of TP53 mutants seem to maintain at least some functionality in the realm of radioreponse (6). The interactions between mutant p53 and PTK2/FAK, and the potential role these interactions have in radioreponse, merit further investigation.

In conclusion, our high-throughput proteomic–genomic method successfully identified novel, targetable biomarkers for radioresistance in HNSCC and validated these biomarkers clinically. Among candidate markers associated with radioresistance, we found that PTK2/FAK blockade sensitized HNSCC cells to radiotherapy and that PTK2/FAK overexpression and amplification were associated with shorter DFS. Taken together, these findings support further evaluation of PTK2/FAK as a marker for identifying patients who are more likely to experience relapse after radiotherapy, and for clinical testing of FAK inhibition in combination with radiotherapy for patients with HPV-negative HNSCC.

Disclosure of Potential Conflicts of Interest

M.D. Story reports receiving commercial research grants from Novocure; speakers bureau honoraria from IBA; and is a consultant/advisory board member for Galera Therapeutics. J.V. Heymach reports receiving commercial research grants from AstraZeneca, Bayer, and GlaxoSmithKline; and is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Exelixis, Genentech, GlaxoSmithKline, Lilly, Novartis, and Synta. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.D. Skinner, U. Giri, I. Yang, S.H. Woo, M.D. Story

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