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IL6 is associated with response to dasatinib and cetuximab: Phase II clinical trial with mechanistic correlatives in cetuximab-resistant head and neck cancer

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Abstract

Objective—Src family kinase (SFK) activation circumvents epidermal growth factor receptor (EGFR) targeting in head and neck squamous cell carcinoma (HNSCC); dual SFK-EGFR targeting could overcome cetuximab resistance.

Patients and methods—We conducted a Simon two-stage, phase II trial of the SFK inhibitor, dasatinib, and cetuximab in biomarker-unselected patients with cetuximab-resistant, recurrent/metastatic HNSCC. Pre- and post-treatment serum levels of interleukin-6 (IL6) were measured by ELISA. HNSCC cell lines were assessed for viability and effects of IL6 modulation following dasatinib-cetuximab treatment.

Results—In the first stage, 13 patients were evaluable for response: 7 had progressive and 6 had stable disease (SD). Enrollment was halted for futility, and biomarker analysis initiated. Low serum IL6 levels were associated with SD (raw p = 0.028, adjusted p = 0.14) and improved overall survival (p = 0.010). The IL6 classifier was validated in a separate trial of the same combination, but was unable to segregate survival risk in a clinical trial of cetuximab and bevacizumab suggesting serum IL6 may be specific for the dasatinib-cetuximab combination. Enhanced *in vitro*

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HNSCC cell death was observed with dasatinib-cetuximab versus single agent treatment; addition of IL6-containing media abrogated this effect.

Conclusion—Clinical benefit and overall survival from the dasatinib-cetuximab combination were improved among patients with low serum IL6. Preclinical studies support IL6 as a modifier of dasatinib-cetuximab response. In the setting of clinical cetuximab resistance, serum IL6 is a candidate predictive marker specific for combined dasatinib-cetuximab. The trial was modified and redesigned as a biomarker-enriched Phase II study enrolling patients with undetectable IL6.

Keywords

HNSCC; Dasatinib; Cetuximab; Interleukin-6; EGFR; Src-family kinases

Introduction

Despite over-expression of the epidermal growth factor receptor (EGFR) in the majority of head and neck squamous cell carcinomas (HNSCC), clinical responses to the EGFR-directed antibody, cetuximab, approximate 10% [1–3]. Unlike colorectal cancer, where activating *KRAS* and *BRAF* mutations predict cetuximab resistance [4], no selection biomarker exists in HNSCC [5,6]. In HNSCC preclinical models, activation of parallel growth factor receptors or downstream signaling nodes circumvents EGFR blockade [7]. Mechanistic identification of such a resistance node could establish a biomarker for clinical selection and/or a rational co-target, addressing an unmet clinical need.

Src family kinases (SFKs) play a key role in both EGFR-dependent and -independent signaling pathways, converging upon STAT3 [8,9]. As shown by our laboratory and others', activation of SFKs leads to EGFR inhibitor resistance [10–12]. Baseline tumoral phospho-Src expression was associated with resistance to the EGFR tyrosine kinase inhibitor erlotinib in patients with operable HNSCC [13]. Dasatinib is a potent multi-targeted inhibitor of at least five selected protein tyrosine kinases/kinase families including several members of the SFKs (SRC, LCK, YES, FYN), BCR-ABL, c-KIT, EphaA2 receptor and PDGFb receptor [14]. Though the SFK spectrum inhibitor, dasatinib has negligible single agent activity in patients with recurrent/metastatic HNSCC [15], the potential for co-targeting EGFR and SFKs has not been exploited. Dual SFK-EGFR targeting could overcome cetuximab resistance by inhibiting EGFR-independent activation of STAT3 by Src. We conducted a Phase II trial evaluating the combination of dasatinib and cetuximab in patients with cetuximab-resistant, recurrent/metastatic HNSCC, after establishing its safety during a Phase I trial enrolling patients with refractory solid tumors [16].

HNSCC is molecularly heterogeneous [17], and responses to dual SFK-EGFR targeting are expected to vary depending upon genetic and biochemical profiles. Thus, we selected mechanistically relevant biomarkers to evaluate for associations with clinical benefit. Circulating cytokines and growth factors such as interleukin-6 (IL6) and vascular endothelial growth factor (VEGF) have been associated with response to cetuximab in HNSCC [16,18]. Activation of the IL6/JAK/STAT3 signaling axis is a known mechanism of acquired resistance to dasatinib [19]. Activation of MET, the receptor for hepatocyte growth factor (HGF), overcomes EGFR blockade in preclinical models of HNSCC and in HNSCC patients

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[12,20,21]. Moreover, serum HGF levels have been associated with resistance to EGFR inhibitors in colorectal and lung cancers [22–24]. Here, we report results for biomarker-unselected patients with cetuximab-resistant, recurrent/metastatic HNSCC treated with dasatinib-cetuximab, and identify serum IL6 as a biomarker of *de novo* resistance to this combination.

Patients and methods

Patients and biologic specimens

Primary inclusion criteria included: recurrent/metastatic HNSCC; progression after previous cetuximab; age 18; Eastern Cooperative Oncology Group performance status <2; adequate end organ function. Primary exclusion criteria included: prior exposure to SFK or EGFR inhibitor other than cetuximab. Blood was collected at baseline and following 6 weeks of treatment and archived tumor tissue was obtained. The protocol was approved by the University of Pittsburgh Institutional Review Board and registered with ClinicalTrials.gov (NCT01488318). Serum from two other protocols was analyzed retrospectively: a phase I trial of dasatinib-cetuximab in refractory solid tumors [16] (NCT00388427) and a phase II trial of cetuximab-bevacizumab, a VEGF-A targeting antibody, in recurrent/metastatic HNSCC (NCT004070810) [25].

Treatment

This single-arm, two-stage, phase II study evaluated the efficacy of dasatinib plus cetuximab in patients with cetuximab-resistant, recurrent/metastatic HNSCC (Supplemental Fig. 1). Cetuximab was dosed at 250 mg/m^2/week following a standard loading dose. Dasatinib 150 mg daily was initiated on day 3 [16]. Cycle length was 3 weeks; patients were treated continuously until progressive disease (PD) or intolerable toxicity.

Evaluations

The primary endpoint was objective response rate (ORR) according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, performed every 2 cycles. Toxicity was graded using the National Cancer Institute CTCAE version 4.0 for toxicity and Adverse Event (AE) reporting [22].

Serum analyses

Blood samples were processed for isolation of sera and stored at −80 °C. Individual enzyme-linked immunosorbent assays (ELISAs) (Quantikine ELISA kits, R&D Systems) for IL6, VEGF, HGF and TGF-α were used to quantify each serum analyte in duplicate. Concentrations between the replicates varied by less than 10% and mean values were used for analysis. Analyte levels below the limit of detection (LOD) were set to one-half the LOD for statistical analyses.

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Human papilloma virus (HPV) status and baseline tumor tissue expression of pSFK and pSTAT3

In situ hybridization (ISH) for HPV DNA was performed as previously described [18]. Tissues were evaluated by immunohistochemistry (IHC) for pSFK and pSTAT3 expression using anti-Phospho-SFK PY-416 (PK1109 1:50; Calbiochem) antibody and anti-Phospho-STAT3 PY-705 antibody (D3A7 1:500; Cell Signaling Technology). Tumor staining was semi-quantified using the Aperio imaging system. IHC scores were calculated as the product of average staining intensity (0–3) and the proportion of positive tumor cells (range 0–300). At least 8 areas all greater than 2 mm^2 were scored for each tumor. While the pSFK immunogen is a synthetic phosphopeptide corresponding to amino acids surrounding the tyrosine 416 phosphorylation site of Src, it is possible that this antibody may also recognize other SFKs phosphorylated at equivalent sites.

Cell line IL6 quantification

3×10^4 cells were plated in each well of 24-well plates and allowed to adhere overnight; media was refreshed the following day. After 24 h media was collected, centrifuged for 5 min at 5000*g*. IL6 levels in cell supernatants were determined using the Human IL6 Quantikine ELISA kit for PE/CA-PJ49, CAL27, CAL33, SCC9, BICR56 and FaDu HNSCC cell lines and cultured tumor-associated fibroblasts (TAFs). IL6 concentration is represented as the average of triplicate samples. TAFs were propagated from HNSCC patient tumors. Cell lines were authenticated by genotyping with a multiplex STR assay (Genetica) within 3 months of performed studies.

Viability assays

1.5×10^4 cells were plated in each well of 24-well plates and allowed to adhere overnight. Cells were treated with vehicle, dasatinib, cetuximab, or dasatinib plus cetuximab at indicated concentrations for 96 h in complete media. Cell viability was assessed by crystal violet assay; briefly, cells were washed once with PBS, fixed with 96% ethanol for 10 min and stained with 0.1% crystal violet solution for 30 min. Stain was solubilized with 1% SDS and absorbance at 570 nm quantified. To evaluate the effects of IL6 on cell viability, treatment conditions included supernatants from TAF cultures (high IL6 conditions) with or without neutralizing IL6 antibody (100 ng/ml, AB-206-NA, R&D Systems).

Immunoblotting analyses

Cell lysate preparation and immunoblotting were performed as previously described [12]. Proteins and phospho-proteins included: P-EGFR PY-1068 (1:1000, #2234, Cell Signaling Technology), EGFR (1:1000, #sc-03, Santa Cruz Biotechnology), P-STAT3 (1:1000, #9131, Cell Signaling Technology), STAT3 (1:1000, #4904, Cell Signaling Technology), IL6 (1:500, #sc-28343, Santa Cruz Biotechnology), IL6 receptor (IL6R) (1:100, #sc-661, Santa Cruz Biotechnology), and β-actin (1:10,000, #MAB1501, Millipore) or β-tubulin (1:2000, #sc-9104, Santa Cruz Biotechnology).

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Statistical methods

The primary endpoint of the trial was ORR; secondary endpoints were progression-free survival (PFS) and overall survival (OS). A Simon two-stage design was employed [26] whereby ORR would be determined following accrual of the first stage and the trial stopped for futility if no responses were observed. Levels of candidate biomarkers were tested for association with response using a two-tailed Wilcoxon test or Kruskal-Wallis test. Test results report both the raw and false-discovery adjusted p values. Significance of changes in analyte levels from baseline was tested using the sign rank test. OS was estimated from the time of treatment initiation to the time of death, or censored at last follow-up. Differential OS by candidate biomarker level (high versus low based on the median value) were evaluated using Kaplan Meier curves and log rank tests. P values were two-sided. *In vitro* studies were analyzed using ANOVA. The joint effect of IL6 and performance status was evaluated with robust rank-based two way analysis of variance [27].

Results

Patient and disease characteristics

Fourteen patients were enrolled between February 2012 and April 2013. Baseline demographics and tumor characteristics are summarized in Table 1. Patients were mostly male with ECOG PS 0-1 with a median age of 62 years. The oropharynx and oral cavity were the most common primary tumor sites. Four of 14 patients had HPV-positive disease as classified by p16 IHC and/or ISH for high risk HPV DNA. As presented in Table 2, all qualifying progression events had occurred either during or within 6 months of prior cetuximab (median 1.9 months; range 0–5.6 months) indicative of cetuximab resistance.

Treatment administration and toxicity

The median duration of protocol treatment was 1.6 months (range 1.2–8.4 months). Thirteen of 14 patients discontinued protocol treatment due to PD or clinical deterioration. One patient with clinical benefit withdrew due to transportation concerns.

All 14 patients were evaluable for toxicity (Table 2). The most common toxicity was acneiform rash, a class effect of EGFR inhibitors. Dasatinib-related toxicities, including infection, pleural effusion, and edema, occurred at expected grades and frequencies. Notably, the edema was facial, a unique pattern that has been observed in patients with recurrent/metastatic HNSCC when exposed to mTOR inhibitors [28].

Efficacy

Thirteen patients were evaluable for the primary endpoint of ORR. Best response to treatment was stable disease (SD). Five patients had SD (36%) and 8 had PD (57%) as best response, thus the study was halted due to the pre-specified futility rule. Median PFS was 1.7 months (90% CI, 1.4–3.9 months) and median OS was 5.1 months (90% CI, 4.2–11.5 months). However, a subset of patients was observed to experience prolonged clinical benefit despite established cetuximab resistance, prompting our search for mechanistic biomarkers associated with prolonged benefit. The patient with longest duration of clinical benefit (8.4 months) had HPV-positive head and neck squamous cell carcinoma of unknown

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primary (as established by both HPV DNA and p16 status). In first line, he was treated with cisplatin, docetaxel, and cetuximab followed by cetuximab maintenance and had progressed during cetuximab maintenance. The patient with the second longest duration of clinical benefit (5.6 months) had HPV-negative larynx cancer with both local and distant pulmonary progression 4.9 months following salvage cetuximab-radiosurgery.

Improved disease control among patients with low serum IL6

We selected serum molecular correlates for analysis based on clinical and/or preclinical data supporting their role as predictive indicators for response to cetuximab [12,16,20,21]. Thus, IL6, TGF-α, HGF and VEGF levels were quantified in baseline ($n = 14$), 6-week ($n = 11$) and PD ($n = 2$) serum specimens. Differences in baseline levels of each biomarker by clinical outcome (SD versus PD) for 13 patients with RECIST treatment response data were evaluated (Fig. 1 and Supplemental Table 2). Of four baseline blood analytes and 4 paired differences, only baseline IL6 serum levels were associated with clinical benefit; patients with SD had a median baseline of 5.0 pg/ml (Interquartile range (IQR) 0–6.4) compared to 34.0 pg/ml (IQR 13.0–59.4) in those with PD (raw $p = 0.028$, adjusted $p = 0.14$). Baseline IL6 still differed by clinical response when adjusting for performance status ($p = 0.0246$). Levels of serum analytes did not change significantly 6 weeks following baseline (Supplemental Fig. 2). Two patients with low baseline IL6 levels experiencing prolonged clinical benefit had serum collected at PD. At time of PD, IL6 levels in these patients had risen 4.7- and 6.5-fold relative to baseline and were numerically comparable to baseline IL6 levels in patients who had PD as best response (Supplemental Fig. 3).

Correlations among baseline serum markers were performed for IL6 only (IL6-HGF, IL6-TGF-α, IL6-VEGF). Serum IL6 levels were highly correlated with baseline HGF levels (Rho = 0.78, $p = 0.0008$). IL6 and VEGF were moderately correlated (Rho = 0.58, $p = 0.0304$) but did not reach the Bonferroni threshold of <0.016 for the 3 comparisons.

To independently evaluate our IL6 finding, we measured IL6 levels in available baseline serum samples ($n = 15$) collected during our previous phase I trial evaluating dasatinib-cetuximab in advanced solid malignancies [16]. Consistent with the phase II results, baseline IL6 levels were lower among patients with SD as best response compared to patients with PD ($p = 0.0184$) (Fig. 2A, left panel). Patients with SD had a median baseline of 0 pg/ml (IQR 0–2.1) compared to 9.9 pg/ml (IQR 5.1–22.6) in those with PD. To explore whether serum IL6 was simply prognostic in patients with advanced HNSCC, we next analyzed available baseline serum samples ($n = 20$) from patients with cetuximab-naïve, recurrent/metastatic HNSCC treated on a phase II study of cetuximab-bevacizumab [25] and found no association between baseline IL6 levels and clinical benefit ($p = 0.743$) (Fig. 2A, right panel). In this study, patients with PR, SD and PD had median baseline IL6 values of 59.2 pg/ml (IQR 32.2–86.2), 8.3 pg/ml (IQR 6.8–13.5) and 17.6 pg/ml (IQR 5.0–30.6), respectively.

A classifier based on serum IL6 was associated with OS

OS provides an assessment of efficacy in advanced solid tumor malignancies, including HNSCC, that is independent of the interval of response evaluation. We selected the median

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serum IL6 value among the 14 clinical trial patients as a data-independent cutoff for classification. The 7 patients with IL6 above the median of 11 pg/ml had a median survival of 2.1 months. The 7 patients with IL6 below the median had median survival of 11.5 months including a long term survivor at 35 months. This difference was significant by a log rank test with p = 0.0101 (Fig. 2B, left panel). To validate this finding, we applied the same classifier to 23 patients on the phase I trial of dasatinib-cetuximab with available serum IL6. This IL6 classifier was able to identify high and low risk groups using the 11 pg/ml cut-off with median OS of 4 and 20 months respectively (log rank p = 0.0096, Fig. 2B, middle panel). However, when the same median-classifier was applied to the 3rd clinical trial of cetuximab-bevacizumab, the high and low risk groups had similar OS of 5.6 and 6.7 months, respectively (log rank p = 0 0.677, Fig. 2B, right panel). The lack of discriminatory ability of the same classifier could be explained by the heterogeneity of the clinical trial populations. Alternately, the classifier may be specific for the dasatinib-cetuximab combination, suggesting the possibility of a predictive rather than prognostic IL6 model.

Archival tumor specimens

Archived tumor tissue was available for 8 patients, 7 of whom had response evaluations. Because EGFR expression and cetuximab response are not generally correlated, we evaluated tumor levels of pSFK, the target of dasatinib [16], as well as pSTAT3, an oncogenic transcription factor that can be activated in pSFK-dependent or pSFK-independent fashion. Although pSFK and pSTAT3 expression levels in archived tumors varied, neither differentiated patients with SD versus PD (p = 0.67 and p = 0.57 respectively) (Supplemental Fig. 4). Furthermore, archival tumor pSTAT3 and baseline serum IL-6 were not correlated (Spearman correlation coefficient = −0.19, p = 0.651). Given the disparity in time points of tumor vs. baseline blood collection, additional tumor endpoints were not pursued due to the likelihood of false discovery in this small sample size.

Combined dasatinib-cetuximab enhances HNSCC cell killing in vitro compared to either single agent

To explore the function of IL6 *in vitro* in the context of response to dasatinib-cetuximab, we evaluated secreted IL6 levels and tumor protein expression of IL6, IL6R, total and activated EGFR and STAT3 in a panel of 7 HNSCC cell lines (Supplemental Fig. 5). PE/CA-PJ49 (PJ49) cells were identified as expressing high levels of IL6 and CAL33 cells as expressing low levels of IL6 by both assays, and these cell lines were selected for further evaluation. Both cell lines expressed intermediate levels of total and phosphorylated EGFR and STAT3. IL6 high-expressing PJ49 cells exhibited sensitivity to cetuximab treatment alone, a significant 51.1% decrease in cell proliferation (Fig. 3A), while IL6 low-expressing CAL33 cells were resistant to cetuximab *in vitro* (Fig. 3B); therefore, response to cetuximab alone was not associated with low IL6 levels in these selected cell lines. Furthermore, PJ49 and CAL33 cells demonstrated similar dose-dependent decreases in cell viability to dasatinib treatment alone *in vitro* (Fig. 3). Importantly, both PJ49 cells and CAL33 cells demonstrated enhanced killing with combined dasatinib-cetuximab treatment compared to single agent treatment (Fig. 3). For example, 30 nM dasatinib alone resulted in a 38.7% and 26.2% decrease in proliferation in PJ49 and CAL33 cells, respectively. The addition of cetuximab to 30 nM dasatinib further inhibited cell proliferation in PJ49 and CAL33 cells to 71.29%

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and 52.3%. These results indicate that HNSCC cells that are unresponsive to cetuximab alone (CAL33) may respond to combined dasatinib-cetuximab treatment. While these cell lines originated from tongue squamous cell cancers that were HPV-negative and the clinical cases do not represent this subset, these cell lines still provide a model system to study the relevance of IL-6 to the combination treatment.

IL6 in conditioned media abrogated enhanced dasatinib-cetuximab HNSCC killing in vitro

We next determined whether IL6 modulates response to combined dasatinib-cetuximab. CAL33 cells, which have low endogenous levels of IL6 secretion, did not demonstrate altered response to dasatinib-cetuximab with the addition of supplemented recombinant IL6 to levels observed in high IL6 expressing cells (data not shown). Since it is possible that both autocrine and paracrine IL6 play a role and the contribution of IL6 may stem from the tumor microenvironment rather than the tumor itself, we used TAF media that secreted high levels of IL6 (2643 ± 102 pg/ml) and found that TAF media abrogated the enhanced dasatinib-cetuximab cell killing observed in the absence of conditioned media (Fig. 4A and B). Addition of an IL6 NAb partially restored enhanced dasatinib-cetuximab killing in the presence of TAF conditioned media (Fig. 4C). Together, these data suggest that IL6 likely acts in concert with other secreted factors in the tumor microenvironment to reduce response to dasatinib-cetuximab. Furthermore, we collected cell lysates after 24 h in these treatment conditions and measured pSTAT3 status. As expected, the cell culture environment with high IL6 levels resulted in an overall 2- to 3-fold increase in pSTAT3 tumor expression. Interestingly with combined dasatinib-cetuximab treatment, pSTAT3 levels increased an additional 2-fold in the high-IL6 environment only (Fig. 4B inset) which was reduced to basal pSTAT3 levels upon treatment with the IL6 NAb. These results suggest that inhibition of the SFK and EGFR pathways through combined dasatinib-cetuximab treatment results in a compensatory increase in pSTAT3 through increased IL6 and may explain why HNSCC tumors exposed to low IL6 may respond more effectively to the combination of dasatinib and cetuximab.

Discussion

Preclinical studies show that SFKs mediate both EGFR-dependent and -independent signaling pathways involved in tumor progression, converging upon STAT3 [8,29,30]. Combined SFK and EGFR inhibition is more effective than either agent alone in blocking HNSCC proliferation and invasion [9]. In a recent study by Baro et al., the anti-tumor efficacy of combined cetuximab-dasatinib treatment was cell line dependent [31]. Furthermore, activation of SFKs can lead to resistance to EGFR-targeted therapies [12,19]. We recently reported a randomized, placebo-controlled window trial in patients with operable HNSCC, where baseline tumoral pSrc expression was associated with resistance to erlotinib [13]. Here, we conducted a phase II trial of dasatinib-cetuximab in biomarker-unselected patients with cetuximab-resistant, recurrent/metastatic HNSCC, after establishing the safety and recommended phase II dose in a phase I trial enrolling patients with any solid tumor [16]. The trial was originally conceived as a two-stage Simon phase II design, where at least 1 response among the first 12 response-evaluable patients was required for progression to the second stage, and was halted for futility. Given the intriguing observation

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of a subset of patients with prolonged disease control, despite clear-cut cetuximab resistance, we searched for mechanistically relevant predictive biomarkers. In both the phase I and phase II cohorts, we identified a significant inverse association between baseline serum levels of the potent pro-inflammatory cytokine IL6, the ligand for JAK2 and a key activator of the STAT3 pathway, and clinical benefit as measured by disease control rate and OS. Among phase II patients, the subset with low serum IL6 had extended SD lasting 5–8.4 months, a clinically meaningful duration in a refractory population. One possible mechanism is that increased baseline IL6 may activate EGFR- and SFK-independent STAT3 signaling, bypassing dual blockade.

IL6 is a plausible biomarker of resistance to the dasatinib-cetuximab combination, by initiating the IL6/JAK/STAT3 signaling cascade, mediating tyrosine phosphorylation of STAT3, and thereby bypassing the SFK pathway for STAT3 activation. Indeed, activation of IL6/JAK/STAT3 signaling through a feedback loop is a known mechanism of acquired dasatinib resistance [19] and mechanistically may explain our failure to rescue patients with cetuximab-resistant, recurrent/metastatic HNSCC with the dasatinib-cetuximab combination. Furthermore, IL6/JAK/STAT3 plays a key role in the tumor microenvironment through both autocrine and paracrine IL6 signaling [32], involving contributions from both stromal and immune cells. While IL6 may be a general prognostic biomarker, an indicator of HPV status or a surrogate for an inflammatory signature in recurrent/metastatic HNSCC, as in patients with newly diagnosed HNSCC treated with curative-intent therapy [33–39], our data suggest it is a predictive biomarker in the specific context of dasatinib-cetuximab treatment for patients with cetuximab-resistant, recurrent/metastatic disease. Baseline activation of IL6/JAK/STAT3 signaling may thus confer *de novo* resistance to dasatinib-cetuximab, precluding clinical benefit. As the relationship between serum IL6 and clinical benefit was independent of performance and HPV status, and was not observed in our prior cetuximab-bevacizumab trial [25], serum IL6 may represent a *bona fide* predictive rather than prognostic biomarker.

In addition to IL6, we evaluated HGF, VEGF and TGF-α based on their well-known roles in development and progression of HN tumors and their association with the EGFR pathway [40]. While HGF levels also appeared higher in patients lacking clinical benefit in this study, this increase was not statistically significant. HGF is the ligand for the MET receptor and can activate many of the same signaling pathways as EGFR. Furthermore, dysregulated MET signaling has been linked to HNSCC [41] and is a known mechanism of resistance to EGFR inhibitors [12,42]. Baseline serum IL6 levels were highly correlated with baseline HGF levels, suggesting a potential interaction between the IL6 pathway and the HGF/MET pathway in HNSCC. Cross-talk between these signaling pathways has been previously observed in liver cell lines whereby HGF has been shown to induce IL6 expression and subsequent STAT3 activation through the JAK pathway [43]. We did not observe a correlation between tumor pSFK or pSTAT3 expression and clinical benefit in this study, similar to our phase I study [16]. However, baseline tumors were archived specimens typically collected at initial diagnosis or first recurrence, and may not reflect the tumor status at time of treatment.

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Our *in vitro* studies confirm that IL6 undermines the ability of dasatinib to restore cetuximab response in a cetuximab-resistant cell line. SFK- and EGFR-independent activation of pSTAT3 through increased IL6 is one possible explanation for this finding. Since IL6 alone was not able to modify cetuximab response, additional factors in the tumor microenvironment may work with IL6 to modulate treatment response. In summary, low serum IL6 levels were significantly associated with clinical benefit, including prolonged SD and improved overall survival, in the context of dasatinib-cetuximab treatment on two trials, a phase I study enrolling patients with refractory solid malignancies and this phase II report. Due to the robust and mechanistically plausible association between serum IL6 and clinical benefit, a phase II trial selecting patients with undetectable baseline serum IL6 and cetuximab-resistant, recurrent/metastatic HNSCC is now ongoing. This bio-marker selection approach will allow continued evaluation of a promising treatment in a subgroup lacking other therapeutic options while minimizing enrollment of patients unlikely to respond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2017.03.011.

Abbreviations

EGFR	epidermal growth factor receptor
SFK	Src-family kinase
HNSCC	head and neck squamous cell carcinoma
IL6	interleukin-6
HGF	hepatocyte growth factor
VEGF	vascular endothelial growth factor
IRB	institutional review board
PD	progressive disease

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ORR	overall response rate
AE	adverse events
HPV	human papilloma virus
SD	stabile disease
ISH	in situ hybridization
PFS	progression-free survival
OS	overall survival

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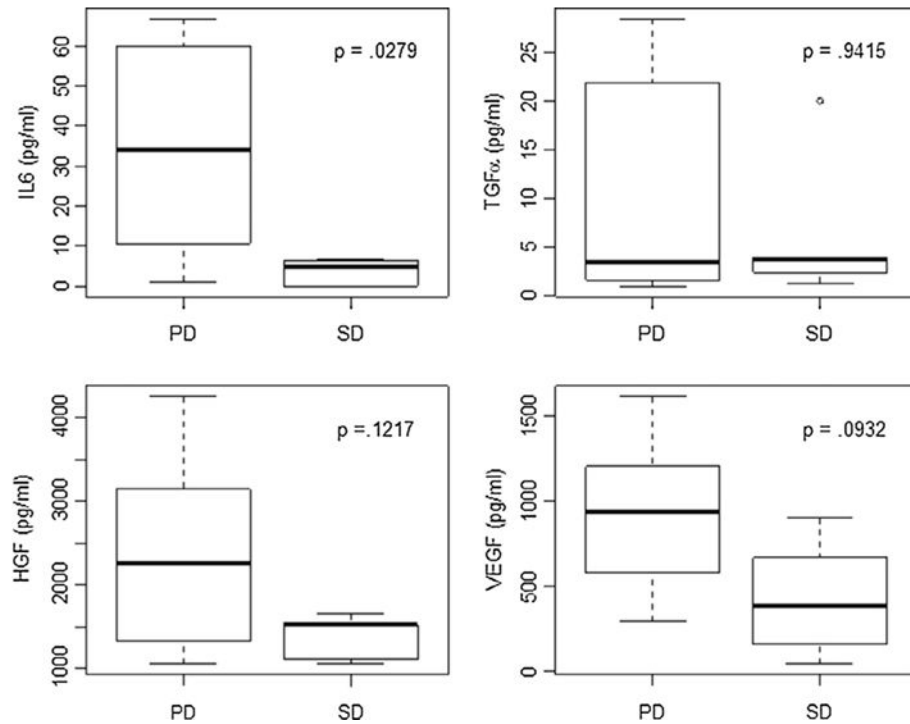

Fig. 1.

Low baseline IL6 serum levels were associated with SD. Baseline serum levels of IL6, TGF-α, HGF and VEGF were evaluated for differences by clinical outcome in 13 recurrent/metastatic HNSCC patients treated on protocol with dasatinib plus cetuximab and evaluated for response to treatment. Eight patients had PD as best response, and 5 patients had SD as best response. Distribution of each analyte by outcome and unadjusted Wilcoxon 2-sided p values are provided.

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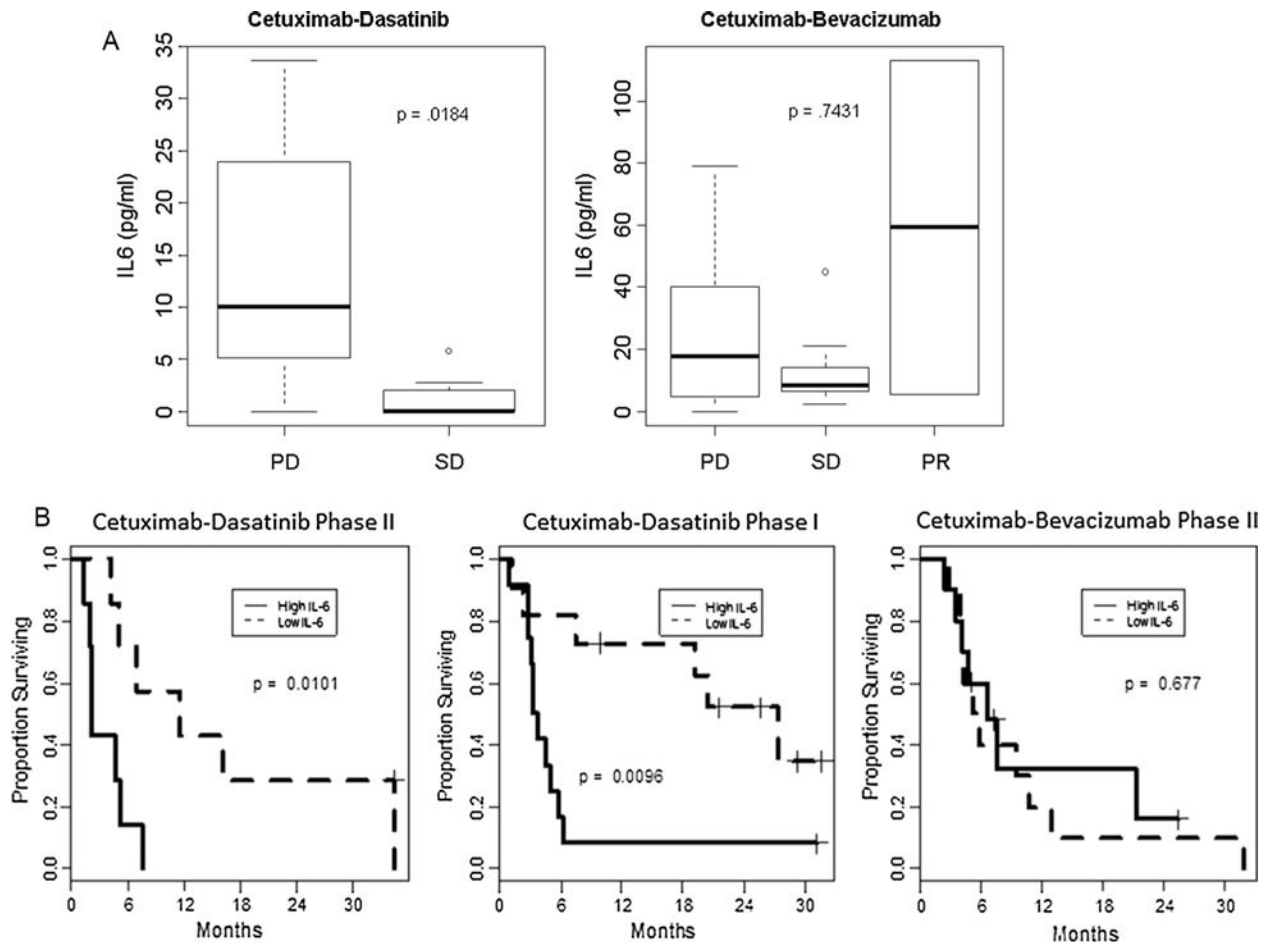

Fig. 2.

Low baseline IL6 serum levels were associated with response and survival to combined dasatinib-cetuximab treatment but not combined bevacizumab-cetuximab treatment. (A) Left panel, In a phase I trial evaluating dasatinib plus cetuximab treatment for advanced solid malignancies. IL6 levels were lower among 7 patients who had SD as best response versus 8 patients who had PD. Wilcoxon 2-tailed p value provided. Right panel, Baseline IL6 levels were measured in 20 available serum samples from a cetuximab plus bevacizumab phase II trial for recurrent/metastatic HNSCC. No difference in IL6 levels was observed across the three clinical response categories (Kruskal-Wallis p = 0.743). (B) Overall survival classified by high and low serum IL6 in 3 clinical trials. Left panel, In the primary trial (phase II dasatinib + cetuximab), patients were divided into high and low IL6 by a median split, ±11 pg/ml. Overall survival was better in the low IL6 group. Middle panel, Overall survival was also improved for patients with low IL6 in the second trial that combined dasatinib with cetuximab. Right panel, In contrast, the same IL6 classifier was unable to distinguish overall survival in a trial of bevacizumab and cetuximab. Survival estimates are Kaplan-Meier estimates; p values are for the log rank test.

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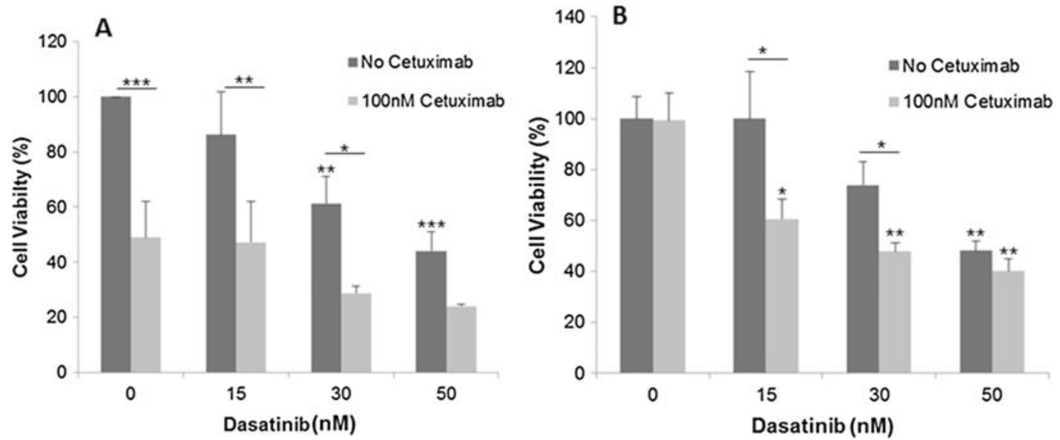

Fig. 3.

Targeting both EGFR and SFK pathways shows enhanced HNSCC cell killing *in vitro* compared to single agent targeting. PECAPJ49 cells (A) or CAL33 cells (B) were treated with 0–50 nM dasatinib in the presence or absence of 100 nM cetuximab for 96 h in 24 well plates. The cells were washed, fixed and stained with crystal violet to determine cell proliferation. Solubilized crystal violet for each well was transferred to a 96-well plate and absorbance was read at 570 nM. Results are the mean ± SD of the average of two independent experiments. ANOVA, $*p < 0.05$; $**p < 0.01$.

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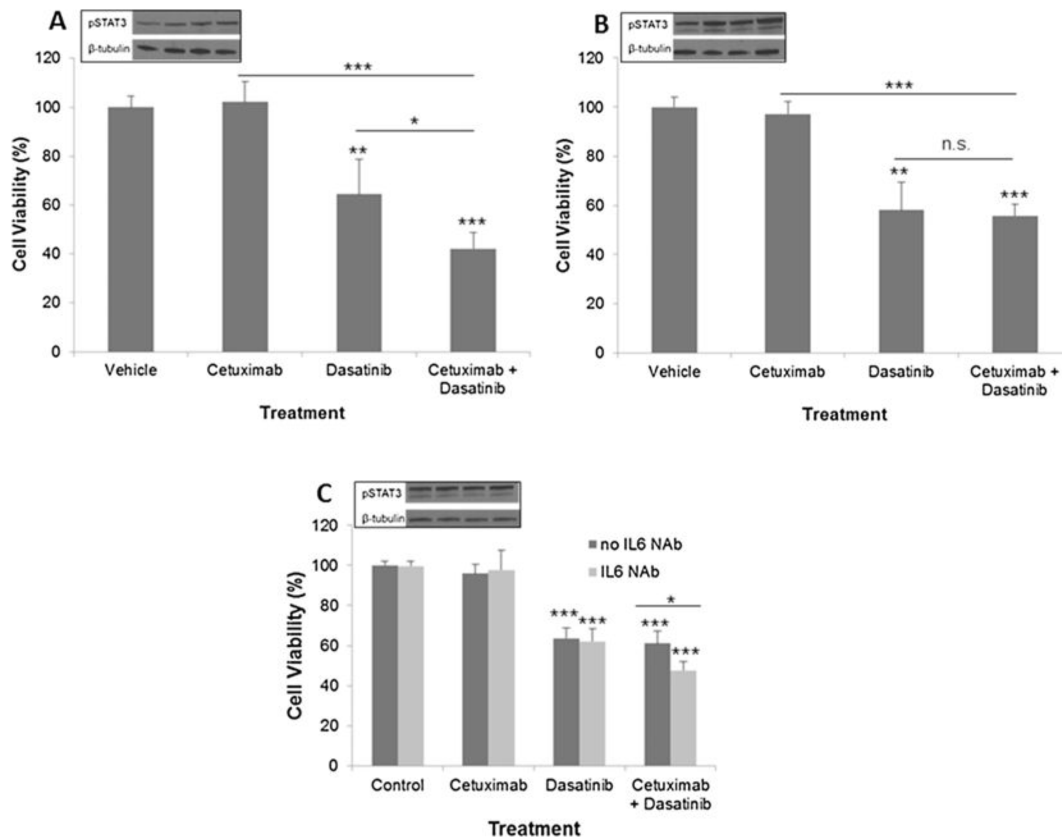

Fig. 4.

IL6 modulates combination treatment response *in vitro*. CAL33 cells were cultured in regular DMEM growth medium (A) or DMEM medium exposed to TAF cells for 48 h (B). Cells were treated with 100 nM cetuximab, 30 nM dasatinib or the combination for 72 h. Cells were then washed and fixed to assess cell proliferation by the crystal violet assay. Results are the average results of 3 independent experiments ± SD. (C) CAL33 cells (bottom) were co-cultured with TAF cells (upper) using cell culture inserts in the presence or absence of IL-6 NAb. Cetuximab and dasatinib treatments were added as in A and B. Results are the average of 2 independent experiments ± SD. ANOVA, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Insets show representative pSTAT3 western blots of CAL33 cells exposed to cetuximab, dasatinib or the combination under each of the culture conditions. Cell lysates were collected after 24 h of drug treatment. Quantitation was performed using Image J software (http://rsbweb.nih.gov/ij/).

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Table 1

Patient characteristics.

Age, years	
Median (range)	62.0 (51.7–72.2)
Sex, N (%)	
Male	12 (86)
Female	2 (13)
ECOG performance status, N (%)	
0	6 (43)
1	7 (50)
2	1 (7)
Cancer Site, N (%)	
Oral cavity	3 (21)
Oropharynx	7 (50)
Hypopharynx	1 (7)
Larynx	2 (14)
Occult	1 (7)
Initial Tumor Stage, N (%)	
T1–2	5 (36)
T3	3 (21)
T4	5 (36)
Tx	1 (7)
Initial Nodal Stage, N (%)	
N0	2 (14)
N1	2 (14)
N2	9 (64)
N3	1 (7)
Tumor HPV Status, N (%)	
Negative	10 (71)
Positive	4 (29)
Cetuximab regimens preceding qualifying progression event,	N (%)
Cetuximab plus definitive radiation therapy	5 (36)
Cetuximab plus salvage radiosurgery	3 (21)
Cetuximab plus chemotherapy	4 (29)
Cetuximab monotherapy	2 (14)
Best response to prior cetuximab-based treatment, N (%)	
Complete response	1 (7)
Partial response	2 (14)
Stable disease	3 (21)
Progressive disease	7 (50)
Inevaluable	1 (7%)
Months between prior cetuximab and documented progression Median (range)	1.9 (0–5.6)

Abbreviations: ECOG, Eastern Cooperative Oncology Group; HPV, human papillomavirus; IHC, immunohistochemistry; ISH, in situ hybridization.

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Table 2

Non-hematologic, treatment-related adverse events.

Rash	
Grade 3	0 (0)
Grades 1–2	6 (43)
Infection	
Grade 3	2 (14)
Grades 1–2	3 (21)
Syncope	
Grade 3	1 (7)
Grades 1–2	0 (0)
Facial Edema	
Grade 3	0 (0)
Grades 1–2	2 (14)
Pleural Effusion	
Grade 3	2 (14)
Grades 1–2	3 (21)