

Increased Expression of HER2, HER3, and HER2:HER3 Heterodimers in HPV-Positive HNSCC Using a Novel Proximity-Based Assay: Implications for Targeted Therapies

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Abstract

Purpose: In other cancer types, HPV infection has been reported to coincide with overexpression of HER2 (ERBB2) and HER3 (ERBB3); however, the association between HER2 or HER3 expression and dimer formation in HNSCC has not been reported. Overexpression of HER2 and HER3 may contribute to resistance to EGFR inhibitors, including cetuximab, although the contribution of HPV in modulating cetuximab response remains unknown. Determination of heterodimerization of HER receptors is challenging and has not been reported in HNSCC. The present study aimed to determine the expression of HER proteins in HPV⁺ versus HPV⁻ HNSCC tumors using a proximity-based protein expression assay (VeraTag), and to determine the efficacy of HER-targeting agents in HPV⁺ and HPV⁻ HNSCC cell lines.

Experimental Design: Expression of total HER1, HER2, and HER3, p95HER2, p-HER3, HER1:HER1 homodimers, HER2:

HER3 heterodimers, and the HER3–PI3K complex in 88 HNSCC was determined using VeraTag, including 33 baseline tumors from individuals treated in a trial including cetuximab. Inhibition of cell growth and protein activation with cetuximab and afatinib was compared in HPV⁺ and HPV⁻ cetuximab-resistant cell lines.

Results: Expression of total HER2, total HER3, HER2:HER3 heterodimers, and the HER3:PI3K complex were significantly elevated in HPV⁺ HNSCC. Total EGFR was significantly increased in HPV⁻ HNSCC where VeraTag assay results correlated with IHC. Afatinib significantly inhibited cell growth when compared with cetuximab in the HPV⁺ and HPV⁻ cetuximab-resistant HNSCC cell lines.

Conclusions: These findings suggest that agents targeting multiple HER proteins may be effective in the setting of HPV⁺ HNSCC and/or cetuximab resistance. *Clin Cancer Res*; 21(20): 4597–606. ©2015 AACR.

Introduction

Head and neck cancer is the seventh most common neoplasm, accounting for 690,000 new cancer cases and 375,000 cancer-related deaths worldwide each year (1). Approximately 90% of head and neck cancers are squamous cell carcinoma (HNSCC). Several variables are associated with an improved prognosis, including, nonsmoker, minimal alcohol consumption, and the absence of comorbid disorders. However, even with multimodality aggressive treatment, the 5-year survival rate of patients with HNSCC is about 40% to 50% (2).

Cetuximab, a monoclonal antibody directed against the epidermal growth factor receptor (EGFR) is the only molecularly

targeted agent approved for the treatment of HNSCC. Despite ubiquitous EGFR expression in HNSCC tumors, the addition of cetuximab to radiation or chemoradiation has resulted in limited benefit to date for the majority of HNSCC patients. EGFR expression, generally assessed by immunohistochemistry (IHC), or EGFR gene amplification determined by fluorescence in situ hybridization (FISH), have not been shown to serve as a reliable predictive biomarkers for EGFR-targeted therapy, including cetuximab, in HNSCC (3).

VeraTag is a proximity-based assay designed to quantify protein expression and dimerization in formalin-fixed, paraffin-embedded (FFPE) tissue specimens. VeraTag has been validated as a method to measure total HER2, HER2 homodimers, or p95HER2 expression in breast cancer (4). VeraTag-determined protein expression levels correlated with IHC results and were reported to serve as a predictive biomarker for HER2-targeted therapy in breast cancer (5). Targeting other members of the HER family, including HER2 (ERBB2) and HER3 (ERBB3), has been shown to enhance responses to EGFR inhibitors in HNSCC preclinical models (6). p95HER2 (p95) is a truncated form of HER2 that lacks the trastuzumab binding domain and contains a hyperactive kinase domain. We previously reported that p95HER2 mediates cetuximab resistance in preclinical cancer models (6).

HPV has emerged as an important cause of an increasing proportion of HNSCC in the United States. HPV⁺ HNSCC demonstrate a more favorable prognosis, regardless of treatment,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-14-3338

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Translational Relevance

Disease heterogeneity as well as *de novo* and acquired resistance to therapy presents a challenge for the treatment of head and neck squamous cell carcinoma (HNSCC). The epidermal growth factor receptor (EGFR)/HER1 is a validated therapeutic target in HNSCC, although response rates to the FDA-approved agent cetuximab are modest and predictive biomarkers are lacking. HER2 and HER3 overexpression and activation in HNSCC are associated with resistance to cetuximab therapy. The incidence of human papilloma virus (HPV)⁺ HNSCC is rapidly rising, and it is increasingly recognized as a disease distinct from HPV⁻ HNSCC. Here, we report significantly elevated levels of HER2, HER3, and activated HER2/HER3 in HPV⁺ HNSCC as well as increased sensitivity of HPV⁺ HNSCC to dual anti-EGFR/HER2 therapy. These results suggest that the subset of HPV⁺ HNSCC patients may benefit from therapeutic agents that target multiple HER family members.

including cetuximab-containing regimens (7). The relative contribution of EGFR expression and signaling to HPV⁺ HNSCC development and progression (compared with HPV⁻ HNSCC) is not well understood. HPV oncogenes have not been shown to modulate the anti-EGFR antibody responses in HNSCC (6, 8). Others have found that EGFR is selectively overexpressed in HPV⁻ HNSCC (9). HER2 and HER3 have been associated with other HPV-associated cancers (10, 11). Only one report to date has compared HER2 expression, among additional biomarkers, in a small number of HPV⁻ and HPV⁺ head and neck precancerous and malignant lesions, and found that HPV⁺ lesions expressed higher levels of HER2 (12). An assessment of HER3 expression or activation in HPV⁺ and HPV⁻ HNSCC has not been reported. Mukherjee and colleagues (13) used the VeraTag assay to investigate the relationship of the HER3/PI3K pathway in breast cancer and found that HER2/HER3 heterodimers and HER3-PI3K complexes were markers of HER3 activity. The objective of the present study was to determine the significance of expression and dimerization of EGFR family members according to HPV status using the VeraTag assay in human HNSCC, including tumors from individuals who received cetuximab-containing therapy on a clinical trial.

Materials and Methods

HNSCC tumors

FFPE pretreatment tissue samples of HNSCC were collected from 88 patients under the auspices of IRB-approved protocols. Thirty-three pretreatment samples were available from individuals treated on a cetuximab-containing induction chemotherapy regimen as previously reported (14). The second cohort consisted of 55 HNSCC tumors collected under the auspices of our institutional Head and Neck Tissue Bank. All patients provided informed consent. Deidentified clinical and pathologic information was obtained from the medical records (Table 1).

Cell lines, cell culture, and reagents

HPV⁺ HNSCC cell lines UM5CC-47, (a kind gift from the University of Michigan, Ann Arbor, MI) and UPCI SCC-90 (a gift

from Dr. Susanne Gollin, Pittsburgh, PA), and HPV⁻ HNSCC cell line Cal33 (a gift from Dr. Gerard Milano, Centre Antoine-Lacassagne, Nice, France) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Inc.) containing 10% heat inactivated fetal bovine serum (FBS; Invitrogen). Cell bullets were thawed in 2010 and prior to their use were genotypically validated by short tandem repeat (STR) using the AmpFISTR Profiler PCR Amplification Kit (Applied Biosystems; Cal33 cells in 2012 and HPV⁺ cell lines in 2011). EGFRvIII-transfected, HPV⁻ HNSCC cells (Cal33vIII) and vector-transfected controls (Cal33control) have been described previously (15). Transfected cells were maintained under selection pressure with DMEM containing 10% FBS and 0.5 mg/mL sG418 (Invitrogen). Cells were incubated at 37°C with 5% CO₂. Afatinib was purchased from Selleck Chemicals. C225 was purchased from the hospital pharmacy.

VeraTag assay

All VeraTag assays were performed on pretreatment FFPE tissue samples of HNSCC described above, cut to 5- μ m thickness and placed on positively charged slides. HER2 overexpression by the HER2 HERmark VeraTag has been analytically validated with cutoffs based on the comparison of routine IHC/ISH HER2 tests with Hermark (4). HER1/EGFR overexpression has not been similarly defined by VeraTag and IHC/ISH, however, total HER1 analytical cutoff could be similarly defined in the same way as HERmark for HER2. P95HER2 and HER3 VeraTag overexpression cutoffs, have been previously described (16). Individual VeraTag methods for detection of HER1, HER2, p95HER2, HER3, HER2:HER3, HER3-PI3K, and pY1829HER2 are described in Supplementary Methods.

EGFR immunohistochemistry

Five- μ m sections were deparaffinized and hydrated with deionized water. Heat-induced epitope retrieval using Borg High PH buffer (Biocare Medical) was performed. Endogenous Peroxidase was quenched using 3% hydrogen peroxide (Fisher Scientific). Blocking was performed using calf serum (Invitrogen) for 10 minutes. Slides were incubated with anti-EGFR mouse monoclonal antibody (Cat# E3138; Sigma) for 60 minutes, washed with TBS buffer for 5 minutes, and then incubated with HRP secondary (Biocare Medical) for 30 minutes. The staining was developed with DAB⁺ substrate Chromagen (Dako) for 5 minutes, and counterstained with Harris Hematoxylin. EGFR expression was semiquantified by positive pixel count v9 algorithm (Aperio), and the protein expression level was represented as a score that combined intensity of staining and the percentage of positive area stained positively. A tumor area with pixel intensity of less than 220 was regarded as positive.

HPV detection

HPV status was determined by IHC for p16. P16 IHC was performed with anti-p16INK4 mAb (BD Pharmingen). Diffuse nuclei and cytoplasm staining pattern with an intensity greater than 2 and a positive area greater than 70% was regarded as p16 positive.

Statistical analysis

All VeraTag assay data were sent to the project statistician (W.E. Gooding) at the University of Pittsburgh. Monogram

Table 1. HNSCC patient clinical and pathologic characteristics

Patient characteristic	N = 88 (%)
Age at diagnosis ^a	
Median	59
Range	23-90
Gender	
Male	68 (79)
Female	28 (21)
Clinical T stage	
1	13 (15)
2	24 (27)
3	10 (11)
4	15 (17)
Recurrent/unknown	26 (30)
Clinical N stage	
0	17 (19)
1	12 (14)
2	32 (36)
3	3 (3)
Recurrent/unknown	24 (27)
Site ^b	
Oral cavity	24 (28)
Oropharynx	30 (35)
Hypopharynx	4 (5)
Larynx	16 (19)
Nasopharynx	3 (3)
Other (parotid, skin, neck)	3 (3)
Histology ^c	
Squamous cell	71 (83)
Basaloid squamous cell	9 (10)
Other	6 (7)
p16 ^d	
Positive	34 (41)
Negative	48 (59)
Cancer type ^e	
Primary	66 (78)
Recurrence	19 (22)

Abbreviations: OC, oral cavity; OP, oropharynx; HP, hypopharynx; L, larynx; SCC, squamous cell carcinoma.

^aTwo patients missing age at diagnosis.

^bTwo patients missing site of tumor.

^cTwo patients missing histology.

^dSix patients missing p16 status.

^eThree patients missing cancer type.

had no role in tissue collection, data analysis or interpretation. VeraTag tumor tissue assay results were compared between two groups (HPV status) with a Wilcoxon test. Association between HER1:HER1 dimer and HER1 or EGFR (by IHC) was evaluated with linear regression allowing a restricted cubic spline fit. Overall survival was calculated from the date of primary disease diagnosis to death or last follow up. The association among overall survival and VeraTag tumor assay results were tested with univariate proportional hazards regression. mRNA expression data were obtained from the The Cancer Genome Atlas (TCGA) Data Portal using R version 3.02. Levels of HER2, HER3, and EGFR mRNA in HPV-positive tumors were compared with levels in HPV-negative tumors using a two-tailed Wilcoxon test.

Afatinib and cetuximab growth and biochemical assays

HNSCC cell lines (Cal33vIII, Cal33control, UMSSC-47, and UMSSC-90) were plated in 96-well plates at a seeding density of 3,000 cells/well, and after 24 hours, cells were treated with varying concentrations of afatinib. Cell viability was analyzed at 72 hours via the MTS assay, using the Cell Titer 96 Aqueous One Solution

Cell Proliferation kit (Promega). The same protocol was followed for treatment with C225 (cetuximab). For biochemistry studies, Cal33vIII, Cal33control, and UMSSC-47 cells were seeded in six-well plates (3×10^5 cells/well) and after 24 hours were treated with increasing concentrations of afatinib. After 24 hours, cells were harvested to obtain cell lysates. Forty micrograms of protein were loaded per lane and separated on 6% sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes with semidry transfer machine (Bio-Rad Laboratories). Membranes were blocked in 5% milk and probed with rabbit monoclonal anti-phospho HER2, phospho-MAPK, phospho-AKT, and rabbit anti-HER2, MAPK, and AKT (Cell Signaling Technology). β -tubulin (Abcam) was used as a loading control. The same protocol was followed for treatment with C225 (cetuximab).

Results

Differential expression of total HER1, total HER2, total HER3, HER2:HER3 heterodimer, and HER3:PI3K complex in HPV⁺ versus HPV⁻ HNSCC

Expression of HER2 and p95HER2 has been previously reported in association with resistance to EGFR-targeted therapy in HNSCC (15). However, the link between HER2 and p95HER2 with HPV status in HNSCC is largely unknown. Expression of HER3 has been associated with resistance to EGFR inhibition in HNSCC (17). HER3 contains an inactive kinase domain and can only form heterodimers with HER2. HER3 also contains recognition sites for the p85 subunit of PI3K. The three activated forms of HER3 include HER2:HER3 heterodimers, phosphorylated HER3 (Y1289), and HER3-PI3K complexes. Reliable antibodies to HER3 are lacking leading to an incomplete understanding of the role of total HER3 and activated HER3 expression in HPV⁺ compared with HPV⁻ HNSCC.

Tumors from 88 HNSCC patients were available for analysis, including 34 HPV⁺ cancers (14@). The majority of tumors analyzed were primary cancers (70 of 88) and 18 of 88 (20%) represented recurrences (Table 1). Thirty-three patients provided specimens in conjunction with being treated on a phase II clinical trial. These patients presented with previously untreated stage III or IVB HNSCC and were enrolled on a protocol where they received induction docetaxel, cisplatin, and cetuximab followed by radiotherapy with concurrent cisplatin, weekly cetuximab, and maintenance cetuximab (14@). The majority of these tumors were HPV⁺ (19 of 33, 70%). Tumors from the remaining patients were obtained mostly from the oral cavity (22 of 55, 40%) or larynx (13 of 55, 24%) and only 27% (25 of 55) were HPV⁺. This second group of patients was generally treated off protocol with surgery, radiation and/or chemotherapy and only a small number (10 of 55) received cetuximab (4 of 10 prior to obtaining the tumor sample and 6 of 10 subsequently received cetuximab). Eighty-five percent of oropharyngeal (OP) tumors with known p16 status, were p16⁺ compared with 9% of other tumor types. Additionally, p16⁺ patients had a significantly more advanced N stage than p16⁻ patients. VeraTag analysis of HNSCC tumors demonstrated higher expression levels of total HER2 ($P = 0.0082$), total HER3 ($P = 0.0007$), HER2:HER3 heterodimers ($P < 0.0001$), and the HER3-PI3K complex ($P = 0.0076$) in HPV⁺ compared with HPV⁻ HNSCC tumors (Fig. 1). In contrast, expression of total HER1 was increased in HPV⁻ tumors ($P = 0.029$; Fig. 1). We also analyzed the VeraTag expression results in

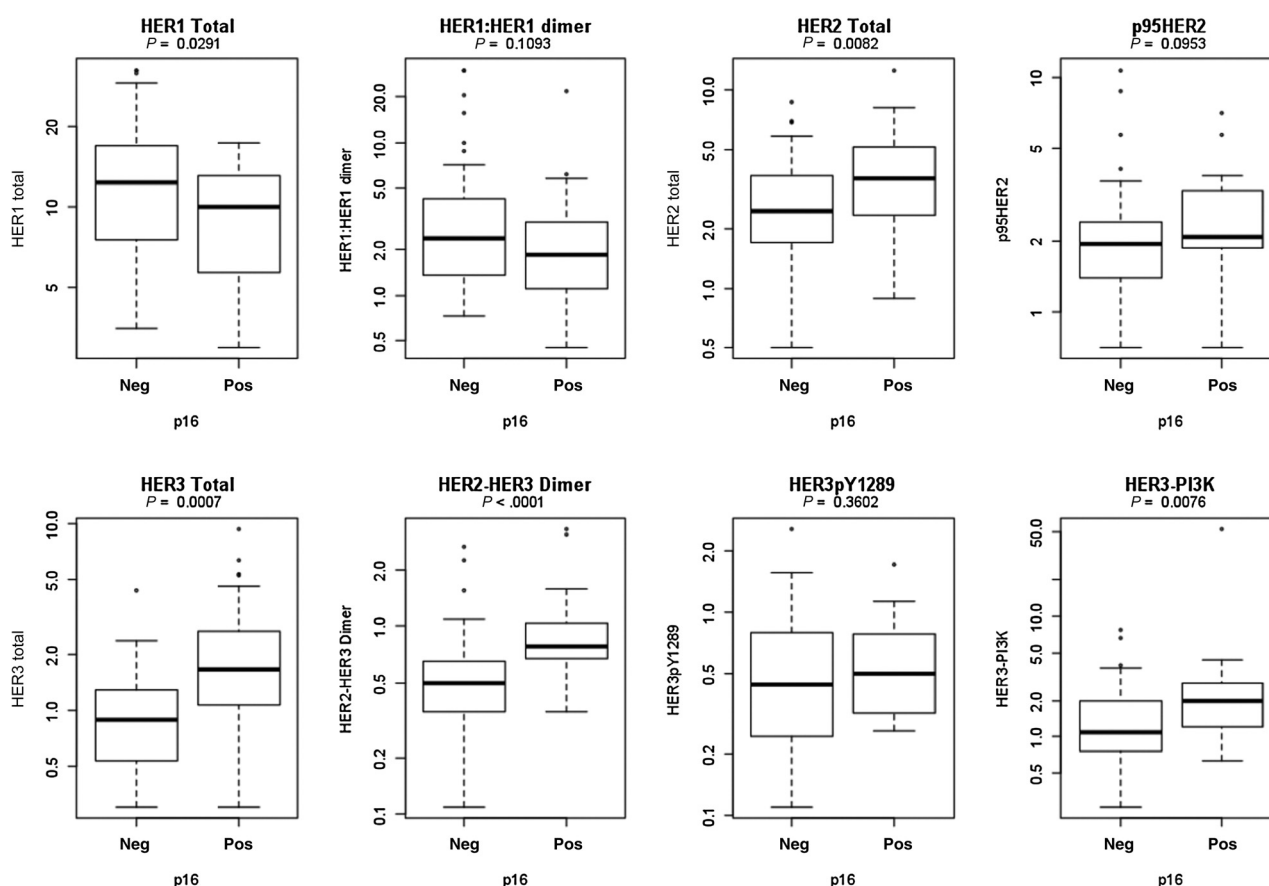


Figure 1. Expression of total HER2, total HER3, HER2:HER3 heterodimers, and HER3-PI3K complexes is significantly higher in HPV⁺ compared with HPV⁻ HNSCC tumors. Expression of total HER1 is significantly lower in HPV⁻ HNSCC. Box-and-whisker plots of VeraTag variables by p16 (HPV) status. *P* values are for a Wilcoxon test comparing p16 positive with p16 negative.

the 27 OP tumors with known p16 status and the findings were unchanged (data not shown).

To compare and validate the differential expression of HER2 and HER3 relative to HPV status in a larger, more homogeneous HNSCC cohort or primary tumors, we examined mRNA expression in TCGA collection of 279 tumors. Although only 36 HPV⁺ (13%) specimens are included in this cohort, all TCGA specimens are derived from primary, previously untreated tumors. As shown in Fig. 2, both HER2 ($P = 0.0006$) and HER3 ($P < 0.0001$) mRNA expression were significantly higher in HPV⁺ compared with HPV⁻ HNSCC in this independent cohort. Additionally, EGFR mRNA expression was significantly increased ($P = 0.001$) in HPV⁻ compared with HPV⁺ HNSCC in the TCGA cohort (Fig. 2). These collective findings suggest that HER2:HER3 heterodimers and signaling are increased in HPV⁺ HNSCC, whereas HER1 (EGFR) is relatively elevated in HPV⁻ HNSCC.

EGFR VeraTag results correlate with EGFR IHC

EGFR expression is routinely assessed by IHC in HNSCC samples. To determine the correlation between expression levels obtained using the VeraTag proximity assay and EGFR IHC, we compared the HER1 VeraTag data with EGFR IHC using linear regression. As shown in Fig. 3, HER1 total protein expression by VeraTag was significantly and positively correlated with HER1

homodimer expression by correlated VeraTag (Fig. 3A). HER1 homodimer expression by VeraTag was also significantly and positively with EGFR IHC (Fig. 3B). These results suggest that either VeraTag or IHC can be considered for assessing EGFR expression in HNSCC samples. Utilization of the VeraTag assay enabled quantitative assessment of receptor homo- and heterodimers and complexes, such as HER3-PI3K, that standard IHC cannot detect. Additional advantages included quantitative assessment of protein expression in FFPE tissues using objective quantification of signal by capillary electrophoresis, and enhanced specificity over IHC due to the requirement of two antibodies in close proximity for signal generation. However, VeraTag is a nonmorphologic-based assay, and so the results obtained do not include histologic details, and there can be difficulty in generating analytical cross-validation of protein complexes in tissues by other methods.

Prognostication and prediction of response to cetuximab-containing therapy based on VeraTag assay

Neither protein EGFR expression by IHC nor EGFR gene amplification has been predictive of clinical responses to cetuximab (18). There was no evidence that a VeraTag assay result was prognostic for overall survival among all 88 patients in the total HNSCC cohort (data not shown).

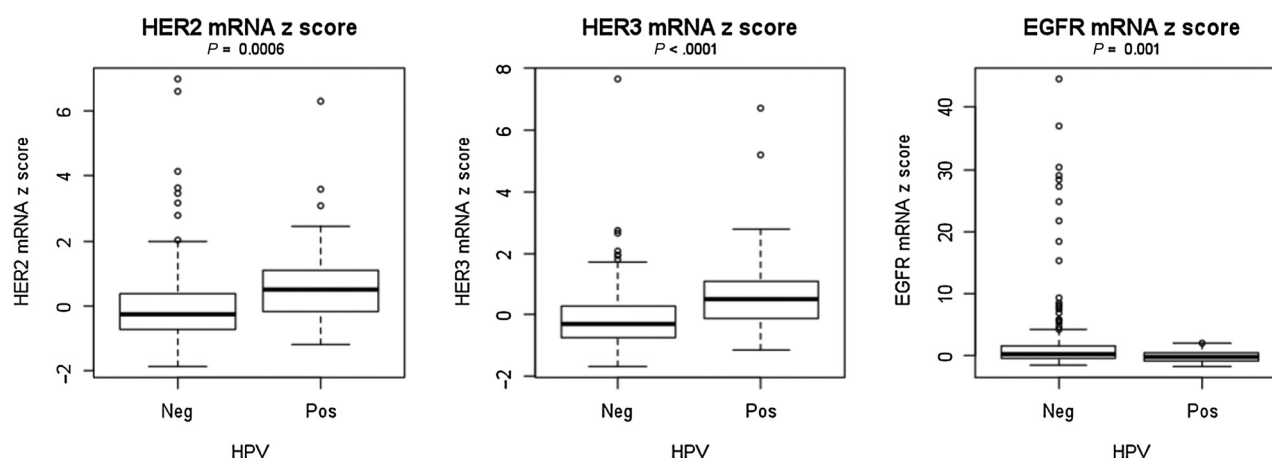


Figure 2.

Expression of EGFR/HER1, HER2, and HER3 in the HNSCC TCGA cohort. Levels of HER2 and HER3 mRNA are significantly higher in HPV⁺ tumors and expression of EGFR/HER1 is significantly higher in HPV⁻ HNSCC tumors as assessed by RNAseq (HPV positive $n = 36$; HPV negative $n = 240$). Box-and-whisker plots of EGFR/HER1, HER2, and HER3 mRNA by HPV status from TCGA. Expression levels have been converted to Z scores, P values are for a Wilcoxon test comparing HPV⁺ and HPV⁻.

HER2 targeting inhibits proliferation of cetuximab resistant and HPV⁺ HNSCC cells

We next tested the hypothesis that HER2 may represent therapeutic targets in HPV⁺ HNSCC using preclinical models. Afatinib is an irreversible dual EGFR/HER2 tyrosine kinase inhibitor that is currently being investigated in HNSCC. We previously reported that afatinib treatment overcomes resistance to cetuximab in preclinical cancer models (6). Although strategies to overcome cetuximab resistance are being widely investigated, HPV-selective treatments are lacking. EGFR variant III (EGFRvIII) is an altered EGFR that results in a truncated ligand-binding domain in HNSCC and other cancers (19). We previously reported that HNSCC models expressing EGFRvIII are resistant to cetuximab (15). To determine if dual HER1/2 inhibition was effective in these models, we compared the effects of afatinib in EGFRvIII and HPV⁺ HNSCC cells. As shown in Fig. 4, afatinib inhibited cell proliferation in cetux-

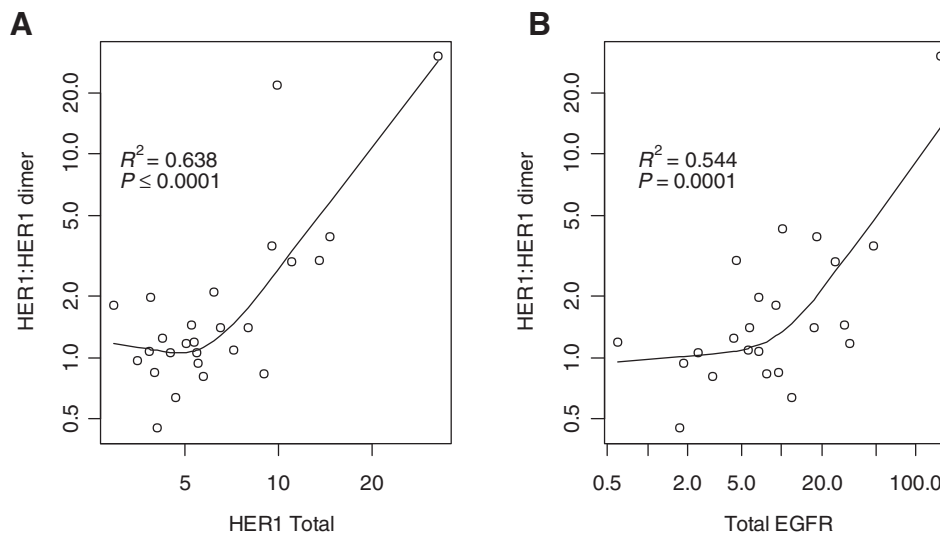
imab-sensitive and cetuximab-resistant HNSCC cell lines (Cal33VC and vIII), and was even more potent in the HPV⁺ cell lines, UM SCC-47, and UPCI SCC-90. These results indicate that proliferation of both cetuximab-resistant and HPV⁺ HNSCC models is inhibited by dual EGFR/HER2 tyrosine kinase inhibitors and pan-HER inhibitors.

Afatinib inhibits activation of the HER2 pathway in a dose-dependent manner in cetuximab-resistant and HPV⁺ HNSCC cell lines

Afatinib targets both EGFR and HER2, but is a more potent inhibitor of EGFR activation (20). Afatinib irreversibly binds to the tyrosine kinase domains of EGFR and HER2 and prevents subsequent phosphorylation of the Ras/Raf/MAPK and PI3K/Akt pathways. Afatinib has shown activity against trastuzumab-resistant breast cancer cell lines (21); however, it has not previously been investigated in cetuximab-resistant HNSCC models.

Figure 3.

EGFR/HER1 VeraTag expression levels positively correlate with IHC results. A, HER1 dimer plotted against HER1 total (HER1/EGFR by VeraTag) and B, total EGFR (HER1/EGFR by conventional IHC). HER1 dimer was positively correlated with both HER1 total by VeraTag and EGFR IHC. Curves and statistics are from a least-squares regression on a 3-knot restricted cubic spline fit for the variables on the x -axis. R^2 reflects the total variation explained by the regression model.



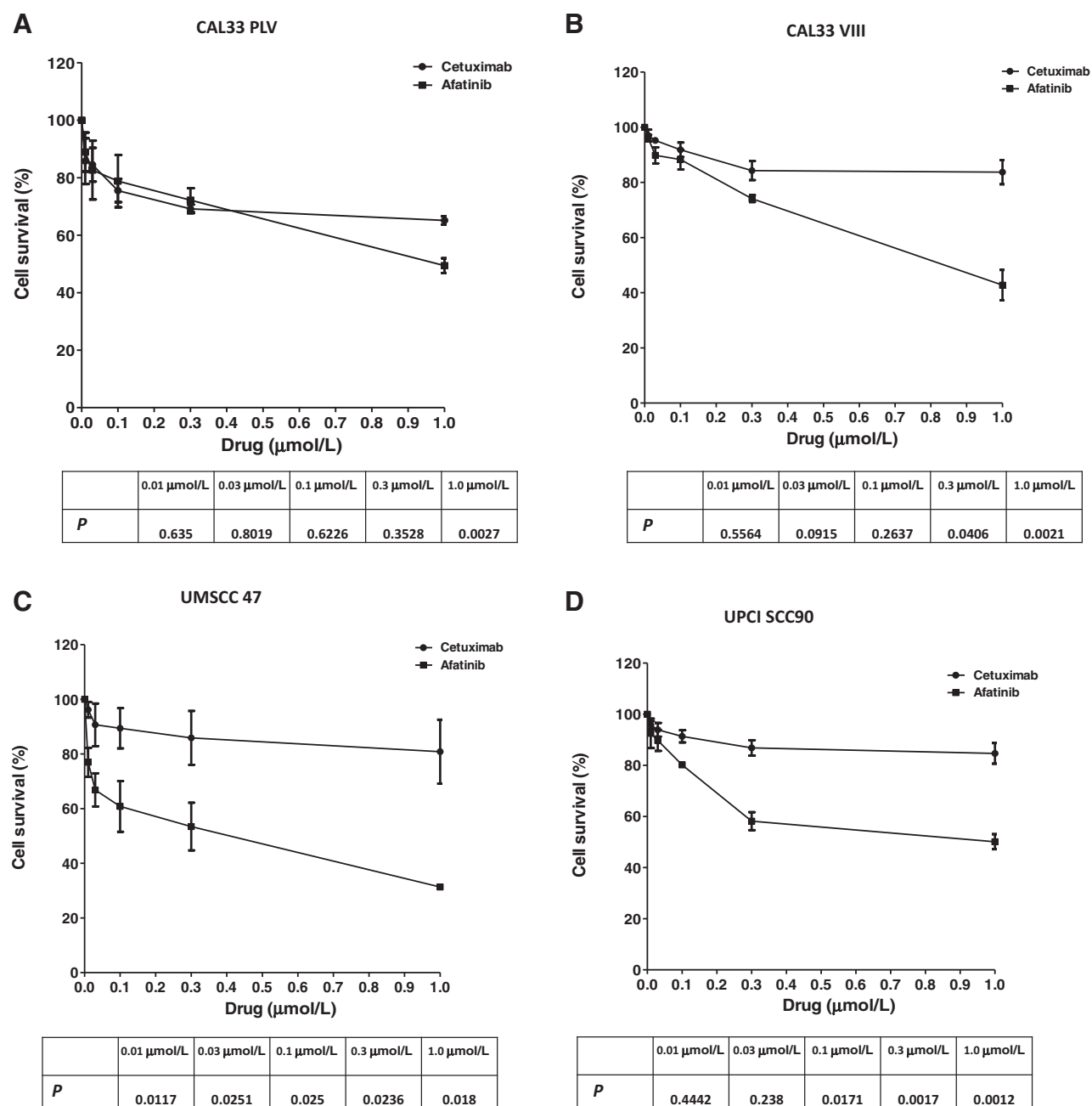


Figure 4.

Afatinib is significantly more effective at inhibiting cell growth in cetuximab-resistant and HPV⁺ HNSCC cell lines. A, Cal33 vector transfected control cells were treated with C225 (cetuximab) or afatinib for 72 hours followed by cell viability assessment. There was no significant difference in the effects of C225 or afatinib in these cells. Cal33vIII (B), UMSSC-47 (C), or UPCI-90 (D) was treated with C225 or afatinib for 72 hours, with afatinib demonstrating significant effects on inhibiting cell viability compared with C225.

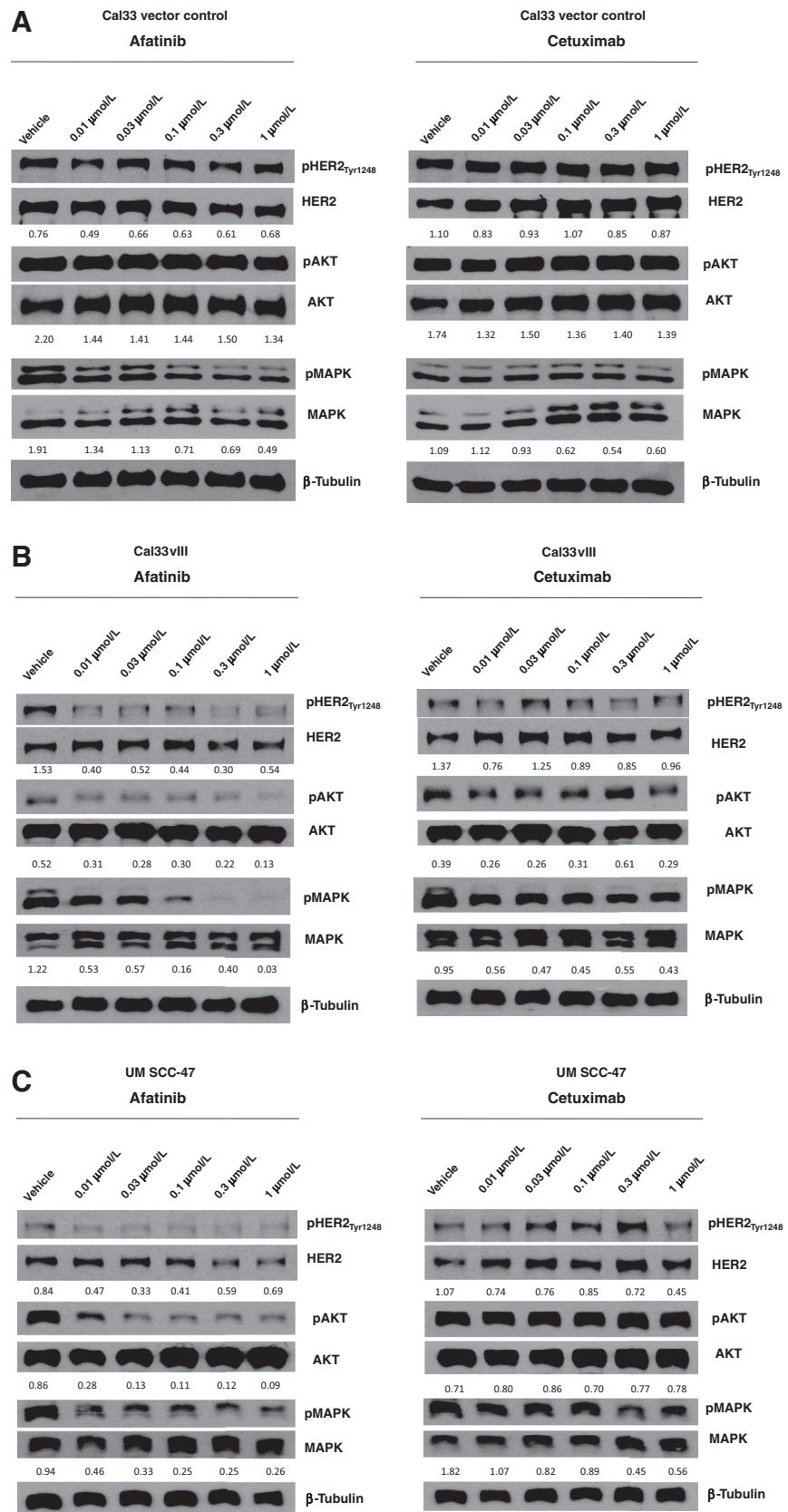
Resistance to cetuximab has been shown to be mediated, at least in part, through alternative activation of the MAPK pathway (22). We sought to determine the effect of afatinib on the expression of HER2 and its downstream targets. Cetuximab-resistant and HPV⁺ HNSCC cell lines express phosphorylation of HER2 at tyrosine 1248. Phosphorylation of HER2 in cetuximab-resistant and HPV⁺ HNSCC cells, as well as Akt and MAPK, appears to be more abrogated by treatment with afati-

nib (Fig. 5B and C). These results suggest that a dual EGFR/HER2 inhibitor abrogates signaling in both cetuximab-resistant and HPV⁺ HNSCC models.

Discussion

HPV infection is responsible for about 5% of cancers worldwide (23), including vaginal, vulvar, and penile cancers. Up to 80% of

Figure 5. Afatinib abrogates EGFR/HER2 signaling in HNSCC models. HNSCC cell lines, including A, HPV⁻ cetuximab-sensitive (Cal33 VC), B, HPV⁻ cetuximab-resistant (Cal33 VIII), and C, HPV⁺ (UMSCC-47, UPCI SCC-90) HNSCC models were treated with increasing concentrations of afatinib. After 24 hours of treatment, cells were harvested to obtain cell lysates. Forty micrograms of protein/lane was subjected to gel electrophoresis and probed with pHER2_{Tyr1248}, pAkt, total HER2, and total Akt. β -Tubulin was used as a loading control. Phosphorylated protein was compared with total protein, and the ratio is listed under each protein band. The experiment was repeated three times with similar results.



oropharyngeal HNSCCs are attributed to HPV infection, and it is estimated that by 2020, HPV infection in the United States will account for more oropharyngeal cancers than cervical cancers (8, 24). HPV⁺ HNSCC is morphologically distinct from HPV⁻ HNSCC. Specifically, non-HPV HNSCCs are typically moderately differentiating keratinizing carcinomas, whereas HPV⁺ HNSCCs are not associated with dysplasia of the surface epithelium, do not keratinize, and demonstrate lobular growth (8). HPV⁺ HNSCC tend to present at an early T stage and advanced N stage and have clinical features associated with a more favorable prognosis: nonsmoker, nominal alcohol exposure, and the absence of comorbid disease. Additionally, patients with HPV⁺ oropharyngeal HNSCC have improved responses to chemotherapy and radiation compared with patients with HPV⁻ tumors (8). Despite the increasing recognition that HPV⁺ HNSCC represents a distinct biologic entity, HPV-selective therapies are lacking.

One study analyzed the correlation of HER2 expression and other biomarkers in a small number of precancerous and malignant head and neck lesions and found that p16 expression was associated with upregulation of HER2/*neu* (12). The impact of HPV status on response to cetuximab therapy is incompletely understood. Others reported that endogenous expression of the HPV oncogenes *in vitro* did not significantly impact response to EGFR inhibition, and response rates to cetuximab in patients with HPV⁺ and HPV⁻ HNSCC were similar (9). However, cetuximab is rarely administered in the clinic as a monotherapy (25), thus evaluating the direct relationship between HPV status and response to cetuximab therapy has been challenging. In the present study, the cetuximab-treated cohort also received docetaxel, cisplatin, and radiation, thereby precluding the ability to assess the role of individual biomarkers in predicting response to cetuximab.

Because overexpression of HER2 and HER3 has been reported in conjunction with cetuximab resistance (26), we evaluated the expression levels of HER proteins in HPV⁺ and HPV⁻ HNSCC. We found that total HER2 and HER3, activated HER2, HER2:HER3 heterodimers, and two activated forms of HER3 were expressed at significantly higher levels in HPV⁺ HNSCC than HPV⁻ HNSCC in two independent HNSCC cohorts. These results are consistent with a prior study that found higher HER2 in HPV⁺ HNSCC lesions (12). While the implication of increased expression levels of HER2 and/or HER3 in HPV⁺ HNSCCs is unclear, our findings support the hypothesis that the cooperation of HPV 16 proteins with HER2 stimulates transformation of normal oral epithelium as well as normal cervical epithelium (27, 28). When normal oral epithelial cells were stably transfected with HPV16 E6/7 and transduced with retroviral DNA containing HER2/*neu*, coexpression of HPV16 E6/7 and HER2 resulted in downregulation of E-cadherin/catenin complex of the normal oral epithelial cells, a complex important in epithelial cell-to-cell adhesion. In contrast, expression of E6/7 or HER2 alone was not sufficient to transform normal epithelial cells (27). P95HER2/611-CTF is a critical target in HER2⁺ breast and gastric carcinomas, where activation of p95HER2 represents a potential mechanism of resistance to the monoclonal antibody, trastuzumab (16). p95HER2 lacks the amino terminal domain, is constitutively active, and able to form heterodimers. We previously reported phosphorylation of p95HER2/611-CTF at Tyr1248, the activation site responsible

for MAPK phosphorylation, in cetuximab-resistant preclinical models (6). These results suggest that activated p95HER2 alternatively activates the MAPK pathway in the presence of EGFR inhibition.

In some cancer types, overexpression or mutation of a biomarker is predictive of response to therapies selective for that target; such is the case in HER2-amplified breast carcinomas or EGFR and HER2 mutated non-small-cell lung carcinomas (NSCLC; refs. 19, 29). In HNSCC, EGFR expression levels and/or amplification have not consistently predicted clinical responses to cetuximab. Both lapatinib and afatinib are dual EGFR/HER2 TKIs and are under active clinical development in HNSCC. We tested the biochemical and phenotypic effects of afatinib in HPV⁺ and cetuximab-resistant HNSCC models to evaluate the efficacy of HER2 targeting with an agent under active clinical development. Afatinib is an irreversible dual EGFR/HER2 tyrosine kinase inhibitor that is approved for the treatment of patients with metastatic NSCLC whose tumors express mutated EGFR. Afatinib has shown activity in HNSCC (30). Interestingly, the HPV⁺ cell lines appeared to be more sensitive to afatinib than the HPV⁻ cell lines, although in the absence of a larger collection of HPV⁺ models it is not possible to conclude enhanced potency in HPV⁺ HNSCC. Sensitivity of both the cetuximab resistant and HPV⁺ models to the dual EGFR/HER2 TKIs suggests that agents that block HER2 may be effective in these important clinical settings. A phase Ib study combining afatinib and cetuximab showed that EGFR inhibitors in conjunction with HER2 inhibitors could overcome T790-mediated erlotinib and gefitinib resistance in NSCLC (31). These results are promising for such combination therapies in HNSCC.

We, and others, have reported alternative activation of the MAPK pathway in the context of cetuximab resistance in HNSCC (6, 22, 26). In the present study, we tested the biochemical effects of afatinib in preclinical HPV⁺ and cetuximab-resistant models. Our results suggest that treatment with afatinib can overcome the activation of the MAPK pathway by inhibiting both phosphorylation of HER2 at the Tyr1248 site and phosphorylation of MAPK. Afatinib was also effective in inhibiting the Akt pathway, which is the primary pathway activated by HER2/HER3 heterodimers. Together, these results show the efficacy of afatinib in cetuximab-resistant and HPV⁺ HNSCC models. While, an EGFR/HER3 targeting antibody, MEHD7945A, was not superior to cetuximab in a randomized phase II study in recurrent/metastatic HNSCC, targeted therapies are most likely to be effective in defined subsets of HNSCC patients (32). The measurements of biomarkers described in this study may be evaluated as predictive biomarkers in patients treated with HER2 and/or possibly HER3-targeted agents.

In summary, our cumulative findings in an *ad hoc* retrospective cohort of HNSCC along with preclinical models suggest that HER2 is overexpressed and activated in HPV⁺ HNSCC where small-molecule inhibitors of HER2 inhibit signaling and cell proliferation. Given the overexpression and activation of HER3 in HPV⁻ HNSCC, these results suggest that agents that target HER2, and/or HER3, may be particularly effective in HPV⁺ HNSCC where selective therapies are lacking.

Disclosure of Potential Conflicts of Interest

W. Huang has ownership interest (including patents) in Labcorp. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors meet criteria for authorship as recommended by the International Committee of Medical Journal Editors (ICMJE). The authors received no direct compensation related to the development of the article.

Grant Support

J.R. Grandis was supported by the NIH under award numbers P50CA97190 and R01DE023685 and the American Cancer Society.

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Received December 23, 2014; revised April 30, 2015; accepted June 13, 2015; published OnlineFirst July 2, 2015.

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