Published in final edited form as:

Mol Cancer Ther. 2016 October ; 15(10): 2486–2497. doi:10.1158/1535-7163.MCT-16-0243.

EGFR and RB1 as Dual Biomarkers in HPV-Negative Head and Neck Cancer

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Abstract

Clinical decision making for human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC) is predominantly guided by disease stage and anatomic location, with few validated biomarkers. The epidermal growth factor receptor (EGFR) is an important therapeutic target, but its value in guiding therapeutic decision making remains ambiguous. We integrated analysis of clinically annotated tissue microarrays with analysis of data available through the

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Disclosure of Potential Conflicts of Interest

B.A. Burtneess is a consultant/advisory board member for Boehringer Ingelheim. R. Mehra is a consultant/advisory board member for Bristol-Myers Squibb and Genentech. No potential conflicts of interest were disclosed by the other authors.

T.N. Beck and R. Georgopoulos are co-first authors of this article.

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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TCGA, to investigate the idea that expression signatures involving EGFR, proteins regulating EGFR function, and core cell-cycle modulators might serve as prognostic or drug response–predictive biomarkers. This work suggests that consideration of the expression of NSDHL and proteins that regulate EGFR recycling in combination with EGFR provides a useful prognostic biomarker set. In addition, inactivation of the tumor suppressor retinoblastoma 1 (RB1), reflected by CCND1/CDK6-inactivating phosphorylation of RB1 at T356, inversely correlated with expression of EGFR in patient HNSCC samples. Moreover, stratification of cases with high EGFR by expression levels of CCND1, CDK6, or the CCND1/CDK6-regulatory protein p16 (CDKN2A) identified groups with significant survival differences. To further explore the relationship between EGFR and RB1-associated cell-cycle activity, we evaluated simultaneous inhibition of RB1 phosphorylation with the CDK4/6 inhibitor palbociclib and of EGFR activity with lapatinib or afatinib. These drug combinations had synergistic inhibitory effects on the proliferation of HNSCC cells and strikingly limited ERK1/2 phosphorylation in contrast to either agent used alone. In summary, combinations of CDK and EGFR inhibitors may be particularly useful in EGFR and p^{T356}RB1-expressing or CCND1/CDK6-overexpressing HPV-negative HNSCC.

Introduction

Head and neck cancer is the sixth most common cancer worldwide; head and neck squamous cell carcinoma (HNSCC) accounts for more than 90% of cases (1). In spite of advances in surgical and radiation techniques, as well as the incorporation of chemotherapy in multimodality treatment designs, the 5-year overall survival (OS) remains at about 50% and has not improved much over the last decades (2). The majority of HNSCC cases are tobacco and alcohol associated, although an increasing number of human papillomavirus (HPV)-positive (HPV⁺) cases are recognized (3). HPV-negative (HPV[−]) HNSCC is generally diagnosed in an older patient population and has significantly worse clinical outcomes compared with HPV⁺ head and neck cancers (3, 4). Part of the challenge of treating HNSCC is managing the immense disease heterogeneity (5, 6). Identification of robust prognostic and drug response–predictive biomarkers are needed to help overcome the current treatment challenges.

Genetic alterations associated with deregulation of the core cell cycle–regulatory machinery are detected in nearly all cases of HNSCC (6, 7). In untransformed cells, interactions between the tumor suppressor retinoblastoma 1 (RB1) and the transcription factor E2F1 typically regulate E2F1 activity. In HPV⁺HNSCC, a viral oncoprotein, E7, inactivates RB1, causing continuous activation of E2F1-dependent transcriptional programs necessary for G₁ to S-phase cell-cycle progression (8). In HPV[−] HNSCC, the tumor suppressor p16 (CDKN2A), which is upstream of RB1, is frequently mutated or deleted, whereas RB1 is rarely genetically altered in this disease subtype (6, 7). p16 regulates activity of cyclin-dependent kinases 4/6 (CDK4/CDK6), which are functionally active in complex with cyclin D (CCND1; ref. 9). CCND1 is one of the most commonly amplified genes in HNSCC (6, 7) and associated with poor survival when expressed at high levels (10, 11). CCND1/CDK4/6 phosphorylate RB1 at a number of different phosphorylation sites, including at T356, which causes inactivation of the protein by forcing its dissociation from E2F1. As reported for

CCND1, high levels of inactivated p^{T356}RB1 have been described as an independent indicator of poor prognosis in HNSCC (9).

In addition to common defects in the core regulatory cell-cycle machinery, HNSCC is typically characterized by constitutive progrowth signaling. EGFR is expressed in more than 90% of HNSCC, and poor prognostic outcomes correlate with increased expression of EGFR or EGFR gene amplification (12–14). This reflects the complementary roles of EGFR, an upstream element of signaling pathways that control cell growth, proliferation and survival, and cell-cycle activity (15–17); expression of additional proteins with functions related to EGFR have also been implicated in the pathogenesis of HPV[−] HNSCC. These include not only additional EGFR family members, such as HER2 (16), but also components of EGFR/HER2 effector cascades that influence survival: PTEN, PI3K, AKT, and BCAR1 (16–18), and proteins that regulate EGFR/HER2 activity, trafficking, and expression, such as CBL, GRB2, and NSDHL (19, 20). Among these, NSDHL (NADP-dependent steroid dehydrogenase-like enzymes), required for the conversion of squalene to cholesterol (21), has recently been shown to have noncanonical function as a regulator of EGFR recycling (19, 20). Depletion or genetic loss of NSDHL significantly disrupts EGFR signaling by reducing total levels of EGFR (22).

The intrinsic heterogeneity of HNSCC (6) is a major challenge to the clinical management of patients. The work described in this report assesses the hypothesis that integrated analysis of commonly altered core cell-cycle regulators (RB1, p16, CCND1, and CDKs) and the highly expressed receptor tyrosine kinase EGFR, as well as associated proteins that regulate their functions, has the potential to identify prognostic biomarkers and perhaps help identify patients most likely to respond to combination treatment with EGFR and CDK inhibitors.

Materials and Methods

Patient cohort, construction of tissue microarrays, and annotation of clinical data

Ninety-nine archived formalin-fixed paraffin-embedded (FFPE) HPV[−] surgical HNSCC specimens collected between 1990 and 2002 were analyzed (FCCC cohort). Institutional Review Board–approved consent forms were signed prior to sample collection. Specimens in which the p16 status was positive or unknown were excluded from the study (23). Five tissue microarrays (TMA) were constructed with tumor cores represented in duplicate and a selection of normal tissue controls. Clinical data were extracted anonymously from the FCCC clinical database (Table 1).

TCGA validation cohort

The Cancer Genome Atlas (TCGA) results shown in this study are based on data generated by the TCGA Research Network (<http://cancergenome.nih.gov/>). TCGA datasets were downloaded from cBioPortal (24) or <http://tcga-data.nci.nih.gov/tcga/tcgaDownload.jsp>. They are listed in Supplementary Table S1 or have been published (6). Survival cut-off points for TCGA data were determined using Cutoff Finder (25), and Kaplan–Meier curves were generated using GraphPad Prism version 6.00 for Mac (GraphPad Software).

Fluorescence IHC

IHC was performed as described previously (9, 23). In brief, tissue sections were blocked with Background Sniper (BS966, Biocare Medical). Antigen retrieval was performed in Tris/EDTA pH 9 Buffer for 20 minutes (S2367, Dako). Sections were incubated with primary antibody (selected on the basis of validation for IHC, or IHC and Western blotting, reported in multiple publications) in Da Vinci Green antibody diluent (PD900, Biocare Medical) at 4°C overnight: EGFR (1:400, 28-0005, Invitrogen; ref. 26), HER2 (1:500, A0485, Dako; ref. 27), PTEN (1:100, 9559, Cell Signaling Technology), NSDHL (1:100, 15111-1-AP, Proteintech Group; refs. 20, 21), and BCAR1 (1:250, ab31831, Abcam). Primary antibodies were visualized using a Cy-5-tyramide signal amplification system (TSA; AT705A, PerkinElmer). In addition to each specific primary antibody, all sections were incubated with anti-pan-cytokeratin antibody (Rabbit 1:400, Z0622, Dako, or Mouse, 1:100, M3515, Dako), followed by Alexa Fluor 555 dye-labeled secondary antibody (Invitrogen). Signals were intensified with Envision reagents (DAKO). Tissue nuclei were stained using Prolong Gold mounting medium (P36931; Molecular Probes) containing 4,6-diamidino-2-phenylindole (DAPI).

Antibodies used for TMAs were validated by Western blot (see below) or immunohistochemical analysis of cell pellets with siRNA depletion of specific antibody targets. For the latter, cells were plated in 12 mL plates with siRNA (see below for details) transfection mixture. After 48 hours, cells were trypsinized and collected by centrifugation in complete media. Cell pellets were resuspended in 10% formalin and fixed for one hour and recentrifuged. The cell pellet was subsequently resuspended in HistoGel (Thermo Scientific, cat. # R904012), embedded in paraffin, and sectioned. Sections were stained as described above, using anti-EGFR antibody (1:400, 28-0005, Invitrogen).

Image acquisition and AQUA analysis

A HistoRx PM-2000 (HistoRx) with AQUASition software was used for automated image capture as described previously (9, 23). A pathologist visually inspected all samples to ensure specimen quality and proper staining; the number of informative samples for each individual marker ranged from 80 to 96 cases (Table 1).

Cell culture, siRNA transfection, drug treatment, and Western blots

FaDu and SCC61 cells were recently obtained from the ATCC and were cultured as recommended by the suppliers. SCC61 (2015) and FADU (2016) cells were sent to IDEXX Bioresearch for authentication. Short tandem repeat (STR) analysis using the Promega CELL ID System (8–9 STR markers plus amelogenin) was performed and verified that the genetic profile of the samples match the known profiles of the two head and neck cancer cell lines. The samples were confirmed to be of human origin, and no mammalian interspecies contamination was detected. Transfection of cells with siRNA was accomplished using DharmaFECT1 (GE Healthcare) at a dilution ratio of 1:100 with serum-free media. Depletion of proteins was accomplished using siRNA SMARTpool (four combined siRNAs per target) from GE Healthcare/Dharmacon: EGFR (gene ID# 1956; cat.# 1027416). Control siRNA (siGL2) was purchased from Qiagen.

For antibody validation, cells were plated in 12 mL plates with lapatinib (LC Laboratories # L-4899) at 0.5 or 1 $\mu\text{mol/L}$ concentrations or the siRNA transfection mixture. After 48 hours, cells were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific; #78501) supplemented with protease/phosphatase inhibitor cocktail (Thermo Scientific; #1861282). Western blotting was performed using standard procedures and was developed using SuperSignal West Pico Stable Peroxidase and Luminol/Enhancer solutions (Thermo Scientific; #1856135 and #1856136). Primary antibodies used were the same as described above, plus anti- β -actin conjugated to horseradish peroxidase (HRP; ab49900) from Abcam. All primary antibodies were used at a dilution of 1:1,000, except anti- β -actin, which was used at 1:50,000. Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies from GE Health-care were used at dilutions of 1:10,000.

For Western blots following drug treatment, 3×10^5 to 5×10^5 cells (FaDu or SCC61) were plated in T150 dishes. Twenty-four to 48 hours after plating, 0.5 $\mu\text{mol/L}$ of lapatinib (S1028, GW-572016, Selleckchem), 0.5 $\mu\text{mol/L}$ of palbociclib (S1116, PD-0332991, Selleckchem), both drugs combined, or vehicle (DMSO) was added to media. Lysates were prepared 2, 24, or 48 hours after drug addition and used for Western blotting following the aforementioned procedures. The following primary antibodies were used: EGFR (2646, ell Signaling Technology), pY1060EGFR (3777, Cell Signaling Technology), HER2 (4290, Cell Signaling Technology), pY1221/1222HER2 (2242, Cell Signaling Technology), p16, (ab201980, Abcam), pT202/Y204ERK1/2 (9101, Cell Signaling Technology), ERK1/2 (4696, Cell Signaling Technology), RB1 (9309, Cell Signaling Technology), and pT356RB1 (ab76298, Abcam).

Cell viability assays were performed using 96-well plates, with 2×10^3 cells per well for FaDu and 4×10^3 cells per well for SCC61. After 24 hours, serially diluted concentrations of lapatinib or afatinib (A-8644, LC Laboratories), palbociclib, or combinations of palbociclib with lapatinib or afatinib (1:1 ratio) were added to cells. After 72 hours of incubation with the inhibitor(s), cell viability was measured with 10 μL /well of CellTiter-Blue (Promega). Optical density readings were made in 570 to 600 nm wavelength range using PerkinElmer ProXpress Visible/UV fluorescence 16-bit scanner. Chou–Talalay analysis (28), to determine the coefficient of interaction value at different effective doses (ED) for combination treatment, was performed using CompuSyn (<http://www.combosyn.com/>).

Statistical analysis

FCCC TMA specimens with a valid read on at least one marker were included in the final analysis. Associations between marker expression level, grade, stage, or survival were determined by choosing an optimal cut-off point using classification and regression trees (CART; Supplementary Table S2; ref. 29) and fit using the rpart procedure in R software (version 3.0.2). Survival curves were generated using the Kaplan and Meier method and tested for significance using log-rank tests. In addition, a multivariate analysis was performed using Cox proportional hazards regression, adjusting for T-stage, N-stage, grade, gender, the patient's age, and the specimen's age (Supplementary Table S3). The relationships between markers and stage/grade were analyzed using Spearman correlation (30). Correlations were presented graphically using the corrplot procedure in R.

Results

Patient characteristics (FCCC cohort)

This study employed TMAs constructed from FFPE specimens of 99 HPV⁻ HNSCC patients (Table 1), previously used to identify expression of p^{T356}RB1 as a prognostic biomarker (9). The majority of specimens originated from the oral cavity (43%), with additional specimens from the tongue (21%), glottis (16%), and oropharynx (11%). Three percent of specimens were from the hypopharynx, and 5% were obtained from other anatomic sites (Table 1). In this patient cohort, high T-stage significantly correlated with poor survival outcomes [T1/2, median overall survival (OS) of 124 months; T3/4, median OS of 43 months; $P=0.032$; Fig. 1A]. The correlation between high N-stage or tumor grade and survival did not reach significance ($P=0.102$ and $P=0.154$, respectively; Fig. 1B and C).

Antibody validation and quantitative immunohistochemical analysis

IHC-optimized antibodies against HER2, PTEN, NSDHL, and BCAR1 were validated by Western blot analyses of HPV⁻ SCC61 HNSCC cell lysates (Fig. 2A and Supplementary Fig. S1A). Comparisons of lysates from cells transfected with control siRNA or siRNA targeting HER2, PTEN, NSDHL, or BCAR1 suggested antibody specificity (Fig. 2A and Supplementary Fig. S1A). As the antibody-targeting EGFR was reported as not optimized for Western blot analysis (31), specificity was confirmed using FFPE cell pellets prepared from HNSCC cells transfected with siRNA-targeting EGFR or with control siRNA (Fig. 2B). Within the general limitations of antibody validation, all antibodies had high specificity for the designated proteins and were subsequently used for AQUA-based assays using the FCCC cohort TMAs.

The dynamic range for the stained tissue was robust for all antibodies (Fig. 2C and Supplementary Fig. S1B; refs. 9, 23, 32). Besides detection at the plasma membrane, cytoplasmic staining of EGFR was substantial, similar to previous reports of lung TMAs stained with the same antibody and assessed by AQUA-based assays (26). To account for the differences in age of the specimens, a time-dependent analysis was performed to verify that antibody target epitopes remained stable over time (Supplementary Fig. S2, as in refs. 9, 33). The variation in time-dependent stability of epitopes was adjusted for in the multivariate analyses for all protein markers (Supplementary Table S3).

Kaplan–Meier analyses indicate low EGFR independently predicts improved OS

CART analysis (29) was used to divide patients treated with either surgery alone or surgery plus radiotherapy into groups with high or low expression of the proteins of interest (Supplementary Table S2). As anticipated (32, 34, 35), Kaplan–Meier analysis indicated lower expression of total EGFR was associated with better survival ($P=0.0173$; Fig. 3A). Further CART analysis of the total cohort indicated that HER2 expression levels did not correlate with statistically significant survival differences ($P=0.828$; Fig. 3A). Multivariate analyses using Cox proportional hazards regression, adjusting for T-stage, N-stage, grade, gender, patient's age, and age of specimens, confirmed significant survival differences based

on EGFR expression [HR (low/high), 2.26; 95% confidence interval (CI), 1.34–3.84; $P=0.002$; Fig. 3A].

An independent validation cohort, based on protein expression data for 186 HPV⁻ HNSCC tumors available through the TCGA (Supplementary Table S1; <https://tcga-data.nci.nih.gov/>), was used to validate the FCCC cohort AQUA-based results for EGFR and HER2. In the TCGA validation cohort, high expression of EGFR again correlated with significantly reduced survival ($P=0.0098$; Fig. 3B); analysis of HER2 protein expression in the TCGA cohort revealed similar non-significant survival trends compared with the FCCC cohort (Fig. 3A and B). The median survival for the FCCC cohort was 30.1 months for cases with high EGFR expression and 83.1 months for cases with low expression; whereas, in the TCGA cohort, the median survival was 13.0 and 32.5 months for cases with high and low EGFR expression, respectively (Fig. 3A and B). The survival differences between the two cohorts is likely due to the predominance of T3/4 cases in the TCGA cohort [TCGA, 140/186 (75%) T3/4 tumors; FCCC, 52/99 (53%) T3/4 tumors; Table 1 and Supplementary Table S1] and possibly variation in the tissue of origin for the analyzed specimens (Table 1). Stratification of the FCCC cohort by T-stage revealed that high expression of EGFR in T3/4 cases indeed more closely matched the survival pattern of the TCGA cohort (14.6 months vs. 62.1 months; $P=0.0177$; Fig. 3C and Supplementary Fig. S3A). In spite of nonsignificant survival differences between groups separated by HER2 expression, independent analysis of only T1/2 tumors indicated that for these cases, low HER2 expression predicted reduced survival (9.1 months vs. 58.1 months; $P=0.0039$; Fig. 3C and Supplementary Fig. S3B), albeit the number of available samples for analysis was limited.

No survival differences were detected for PTEN, NSDHL, or BCAR1 in the complete FCCC cohort of HNSCC patients (Supplementary Fig. S4A). However, low NSDHL expression indicated significant survival benefits in patients with high T-stage disease ($P=0.015$; Fig. 3D and Supplementary Fig. S4B), matching the survival profile for low expression of EGFR in T3/4 cases (Fig. 3C). No proteomic data for NSDHL are currently available through the TCGA network. NSDHL functions in a complex composed of four proteins that must associate for full enzymatic activity: NSDHL, MSMO1, HSD17B7, and C14ORF1 (20). Effective antibodies for the other complex members are not available for IHC; however, integrated analysis of RNA sequencing (RNA-Seq) data for all four members of the NSDHL complex indicated that low expression of any one of the four genes significantly correlated with reduced survival in the TCGA HNSCC cohort (64.78 vs. 28.32 months; $P=0.0376$; Fig. 3E). As anticipated for an enzymatic complex with set stoichiometry of subunits, expression of all four genes significantly correlated with each other (Fig. 3F). Importantly, the NSDHL complex survival data closely resembles the EGFR (TCGA) Kaplan–Meier survival curve (Fig. 3B), as predicted based on the known functional relationship between the two entities (19–22). Analysis of T1/2 and T3/4 tumors stratified by PTEN or BCAR1 expression did not show any survival differences (Supplementary Fig. S4C and S4D).

Integrated consideration of RB1-related proteins in the context of EGFR

We first assessed whether expression of EGFR correlates with the expression of p^{T356}RB1 and RB1 (previously analyzed using the same TMA, with p^{T356}RB1 prognostic for OS; ref.

9), or the expression of any of the other proteins analyzed. In the set of proteins analyzed, expression of EGFR only inversely correlated with p^{T356}RB1 ($P = 0.028$; Fig. 4A). Expression of NSDHL, PTEN, and BCAR1 correlated with one another, but none of the three proteins correlated with EGFR. HER2 expression did not correlate with any of the proteins considered in this study, including EGFR.

To explore the correlation between EGFR and RB1 more extensively, we further analyzed the TCGA cohort data. In the absence of data for p^{T356}RB1, cyclin D1 (CCND1; refs. 36, 37), which interacts with CDK4/6 to impose inhibitory T356 phosphorylation on RB1, was employed as a protein surrogate marker. This analysis showed that normalized protein expression of EGFR inversely correlated with CCND1 protein expression in the TCGA cohort, paralleling the FCCC cohort TMA results for EGFR and p^{T356}RB1 (correlation = -0.43 , $P = 2.96 \times 10^{-7}$; Fig. 4B) and supporting the role of the TCGA dataset as a matched cohort.

Phosphorylation of RB1 by the active CDK4/6-CCND1 cyclin/kinase complex is negatively regulated by p16 (CDKN2A; ref. 9). To further explore the relationship between EGFR and RB1 phosphorylation control, we analyzed mRNA expression data from the TCGA HNSCC cohort. EGFR mRNA upregulation co-occurred with upregulated CDK6 ($P = 9.3 \times 10^{-9}$) as well as upregulated/amplified CCND1 ($P = 0.0075$; Fig. 4C). Low CCND1 expression, functionally similar to low p^{T356}RB1, correlated with highly significant survival benefits in the context of high EGFR expression (65.77 months vs. 13.63 months for high EGFR/low CCND1 and high EGFR/high CCND1; $P = 0.0024$; Fig. 4D). The high EGFR/low CCND1 group even had superior OS compared with the group of patients with low EGFR protein expression (32.5 months; Fig. 3B). Consideration of CCND1 amplification did not significantly impact survival (Supplementary Fig. S5B).

Although very strong elevation of p16 expression is normally considered in the context of HPV⁺HNSCC (38), previous work has shown improved survival for HPV⁻ cases with high p16 expression (39). The TCGA network extensively validates HPV status for all cases, using p16 staining and *ISH*, whole-HPV genome sequencing as well as HPV RNA-Seq (6). As expected, the range of p16 mRNA expression levels was significantly lower for HPV⁻ cases compared with HPV⁺ cases. For HPV⁻ tumors, the median p16 expression was 260.67 (range: 0.42–4,354.66), and for HPV⁺ tumors, the median expression was 2,908.93 (range: 702.42–13,389.08; Supplementary Table S4). Among HPV⁻ HNSCC cases reported in the TCGA, stratification by EGFR mRNA expression and p16 mRNA expression revealed that patients with high EGFR and relatively higher p16 (mechanistically similar to low expression of CCND1, as both high p16 and low CCND1 are associated with reduced inactivating phosphorylation of RB1; ref. 9) had significantly improved survival compared with the group with high EGFR and low p16 ($P = 0.011$; Fig. 4E). In HNSCC cases with low EGFR expression, p16 expression levels did not correlate with survival differences, supporting the potential link between RB1-dependent cell-cycle regulation and high EGFR expression (Supplementary Fig. S5C).

We next assessed expression of the CCND1 partner CDK6 in the context of different levels of EGFR expression. Interestingly, low expression of CDK6 (L; z-scores <0) mRNA

correlated with increasing expression of CCND1 (Fig. 4F), potentially indicating a compensatory mechanism; however, this trend was not seen in cases with medium (M; z-scores of 0–1) or high (H; z-scores >1) mRNA expression levels of CDK6 (Supplementary Fig. S5D). In the context of high EGFR, grouping cases with high CDK6 or high CCND1 expression, both mechanistically related to high phosphorylation of RB1, and comparing this group with cases with medium CDK6 expression, showed significantly improved survival for the group with high EGFR/CDK6 (M) expression (with no compensatory CCND1 expression) versus the group with high EGFR/CDK6 (H/L) expression (with compensatory CCND1 expression; 14.32 months vs. 71.16 month; $P = 0.024$; Fig. 4G).

Inhibition of EGFR, HER2, and CDK4/6

The correlations between EGFR expression and RB1 activity identified above suggested the possibility of important connections between EGFR activity and RB1 cell-cycle regulation. Therefore, simultaneous targeting of EGFR and reduction of inhibitory phosphorylation of RB1 might be therapeutically beneficial. This is a testable model with application of lapatinib or afatinib, both dual inhibitors of EGFR and HER2, in combination with palbociclib, an inhibitor of CDK4/6. Combining these two types of inhibitors in two HPV[−] HNSCC cell models, FaDu and SCC61, indicated significant synergy in terms of reduction of cell viability based on Chou–Talalay analysis (Fig. 5A and B; ref. 28).

Analyzing the signaling interaction of these two different drugs in the cell models mentioned above, it was observed that treatment with lapatinib robustly blocked p^{Y1068}EGFR, p^{Y1221/1222}HER2, and p^{T202/Y204}ERK1/2 expression, without affecting p^{T356}RB1 expression levels (Fig. 5C and D). In contrast, the CDK4/6 inhibitor palbociclib robustly inhibited p^{T356}RB1; however, p^{Y1068}EGFR and p^{Y1221/1222}HER2 increased beyond baseline activity 24 to 48 hours after inhibition of CDK4/6, suggesting potential compensatory activity. The combination of lapatinib and palbociclib very effectively inhibited p^{T356}RB1 as well as p^{Y1221/1222}HER2 and p^{Y1068}EGFR (Fig. 5C and D). Strikingly, this combination also resulted in a deeper and more durable decrease in p^{T202/Y204}ERK1/2, compatible with the much-enhanced reduction in cell viability observed (Fig. 5A and B).

Discussion

This is the first study to assess EGFR and HER2 expression in the context of RB1, p^{T356}RB1, CCND1, and CDK6 in HPV[−] HNSCC (Fig. 5E). Analysis of EGFR and HER2 expression is particularly relevant, given the multitude of therapeutic agents that target these receptors (16), their upstream regulation of CDK4/6 cell-cycle activity (40), and the availability of drugs that specifically target CDK4/6 (41). To date, reliable response-predictive biomarkers have not been established for targeted therapies used to treat HNSCC.

High expression of EGFR has consistently been identified as associated with worse survival in HNSCC (32, 33). This longstanding finding was confirmed in this study (Fig. 2). The paradoxical lack of correlation between EGFR expression levels and response to cetuximab reported in other studies (32, 34, 42) suggests that additional factors, such as cell-cycle regulation and EGFR trafficking, may have to be considered to capture the response-predictive value of EGFR expression. We had hypothesized that low expression or loss of

the tumor suppressor PTEN was a confounding factor in earlier studies of cetuximab, extrapolating from a mechanism linked to erlotinib (EGFR inhibitor) resistance in lung cancer (43). We did not find any correlation between PTEN and EGFR expression (Fig. 4A), which does not rule out the possibility that low PTEN expression provides tumor cells with an advantage in the context of EGFR-targeted therapy. We also did not detect any survival differences based on HER2 expression in the FCCC cohort, except in low T-stage tumors (T1/2), where high HER2 expression correlated with reduced survival (Fig. 3C), nor in the TCGA cohort (Fig. 3), nor did we detect any correlation between EGFR and HER2 expression (Fig. 4A). A limitation on this analysis is the currently limited number of specimens expressing high HER2; further investigation is clearly warranted as more specimens become available. In addition, the prognostic value of HER2 may be dependent on concurrent expression of HER3 and HER4 and on homo- and heterodimerization, aspects beyond the scope of this study, but certainly to be considered in future work, particularly in the setting of low T-stage disease.

Previous studies suggested that regulation of the active recycling and localization of EGFR, often observable within the cytoplasm (Fig. 2C; ref. 26), to the plasma membrane by the EGFR-trafficking protein NSDHL might be relevant (19–22). NSDHL and its functional partners MSMO1, HSD17B7, and C14ORF1 (Fig. 3F) influence endosomal trafficking of EGFR (19). Compared to tumors with high levels of members of the NSDHL complex, in tumors with low NSDHL, the pool of stable, active EGFR may be significantly compromised in the presence of EGFR inhibitors (20). Furthermore, inhibition of the NSDHL complex also targets shuttling of the EGFR dimerization partners HER2 and HER3, and possibly of additional receptor tyrosine kinases also involved in resistance to EGFR inhibitors (20). The recent observation that depletion of NSDHL markedly sensitizes cancer cells to EGFR-targeting drugs strongly supports the potential relevance of NSDHL in terms of EGFR expression in HNSCC (20). Further work is needed to identify the prognostic and therapeutic ramifications of EGFR localizations, which has previously been suggested, particularly in terms of nuclear localization of EGFR (15).

Cell-cycle deregulation is likely to significantly interact with deregulation of EGFR in affecting tumor prognosis (40). HPV⁻ HNSCC cases with low p^{T356}RB1 have improved survival (9), and the same has been demonstrated for low levels of CCND1 (10, 11). In complex with CDK6, CCND1 is directly involved in phosphorylation of RB1 (44). Importantly, EGFR activity had previously been observed to regulate cell-cycle progression via ERK1/2-dependent induction of CCND1 (36, 37). This report shows that combined consideration of p^{T356}RB1 status and EGFR expression may highlight cases with prognostic differences and differences in therapeutic response. While requiring validation, including particularly in animal models of HPV⁻ HNSCC, this observation may prove valuable in selection and stratification of patients for clinical trials of targeted agents.

Complementary to the data for EGFR and p^{T356}RB1, data from the TCGA showed a strong inverse correlation between expression of EGFR and expression of CCND1, the most commonly amplified gene in HNSCC and critical for the phosphorylation of RB1 (Fig. 4B; ref. 6). These findings suggest an important relationship between EGFR and cell-cycle regulators and highlight the need to more closely define this relationship in animal models

and in the clinic. On the basis of TCGA data, cases with high mRNA expression of EGFR in the context of high CCND1 have poor OS compared with cases with high EGFR and low CCND1. In the case of the CCND1 activation partner CDK6, the analysis was complicated by the observation that in at least some cases of low CDK6 (predicted to correlated with improved survival, as seen for CCND1), CCND1 expression was significantly elevated (Fig. 4F). This finding suggests that low CDK6 expression is compensated for, in at least some cases of HNSCC, with overexpression of CCND1 as a mechanism to support cell-cycle activity. Furthermore, this compensatory mechanism recapitulates the reduced survival observed for high CCND1 and for low p16 in the context of high EGFR. It is possible that for cases with high EGFR, it predominantly matters that cell-cycle activity is supported, rather than how it is supported. Future studies are needed to further explore this provocative possibility.

Clinical trials are currently testing combination treatment with an EGFR inhibitor and a CDK4/6 inhibitor in HNSCC (NCT02101034; NCT02499120; ref. 45), underscoring the potential clinical value of biomarkers to select patient subpopulations most likely to benefit from this treatment. Afatinib and lapatinib, both of which broadly target the different proliferative markers analyzed in our study, would likely be more clinically efficacious at lower, less toxic doses if used as a part of combinatorial treatment. Lapatinib targets ERBB family members EGFR, HER2, and HER4 at low concentrations and, although less efficiently, also mutant EGFR (T790M), c-MET, and YES1 (46). It is currently under evaluation in a large clinical trial for HPV⁻ head and neck cancer patients (NCT01711658) and has been approved by the FDA for HER2⁺ breast cancer. Lapatinib has recently been shown to synergize with CDK inhibition in HER2-associated malignancies (40). It was noted in the same study that inhibition of CDK4/6 with abemaciclib resulted in increased activation of EGFR/HER2 signaling, similar to the response to palbociclib observed in HNSCC (Fig. 5C and D). In HNSCC, lapatinib, as monotherapy, did not improve OS or progression-free survival (PFS; ref. 47), emphasizing the need for use of response-predictive biomarkers and consideration of combinations with therapeutic agents, such as palbociclib. Afatinib, FDA approved for treatment of non-small cell lung cancer harboring EGFR mutations, has shown some impact on PFS in HNSCC (45, 47). Overall, the work in breast cancer by Goel and colleagues (40) and this report support the notion that simultaneous targeting of EGFR and CDKs has enormous clinical potential for some patients. The presented work suggests that patient selection for this therapeutic strategy could potentially be optimized using EGFR and p^{T356}RB1 as dual biomarkers.

This retrospective study reports only OS and not disease-specific survival for 99 tumors, a modest sample size. Patients included in the analyzed cohort were not treated with cetuximab, which might have added additional information. Prospective studies are needed to determine the predicative value of EGFR expression and activity and RB1-associated cell-cycle regulators, specifically in the context of EGFR and/or CDK inhibition. It is also important to further validate the full mechanistic relationship between cell-cycle regulators and EGFR using *in vivo* analysis, and ideally incorporating primary patient samples, particularly to validate the potential of EGFR and RB1-associated proteins as response-predictive biomarkers. Data from patients would also be particularly helpful in addressing whether the altered expression of the cell-cycle regulators considered here is a passive

reflection of increased proliferation in some tumors that predicts response to EGFR and CDK4/6 inhibitors, or whether changes in CCND1, CDK6, and p16 expression are specifically induced in a manner separable from general cell-cycle effects. Finally, in spite of clear on-target activity of the tested therapeutic agents in terms of signaling changes, additional off-target effects may also contribute to loss of cell viability. Analysis of pre- and posttreatment tumors could potentially address this point.

In summary, this report provides compelling evidence that RB1-associated cell-cycle regulators are important features modulating the prognostic potential of EGFR-associated proliferative activity in HNSCC. EGFR and p^{T356}RB1 should be explored as prognostic biomarkers, specifically to select patients considered for or actively receiving treatment with EGFR/HER2 and/or CDK inhibitors. We strongly encourage clinical investigation of combination treatment with EGFR and CDK4/6 inhibitors. Patients with high p^{T356}RB1 HPV⁻ HNSCC may benefit substantially from combination treatment with EGFR and CDK4/6 inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the facilities and all colleagues at Fox Chase Cancer Center as well as all the patients and their families and donors who contributed to the Head and Neck Keystone Fund.

Grant Support

This work was supported by U54 CA149147, R21 CA181287, R21 CA191425, and P50 CA083638 from the NIH (to E.A. Golemis), by the Ruth L. Kirschstein NRSA F30 fellowship (F30 CA180607) from the NIH (to T.N. Beck), by NCI Core Grant P30 CA006927 (to Fox Chase Cancer Center), and by funds from the Russian Government to support the Program for Competitive Growth of Kazan Federal University (to I.G. Serebriiskii).

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Figure 1. Kaplan–Meier survival analysis for tumor grade and stage. **A–C**, T-stage (**A**), N-stage (**B**), and grade (**C**). Well/Mod. Diff., well and moderately differentiated tumors; Poor/Undiff., poorly differentiated and undifferentiated tumors.

Figure 2.

Validation of antibodies. **A**, Western blots for the indicated protein markers after siRNA depletion of HER2 or NSDHL. **B**, representative immunofluorescent microscopy images of SCC61 cell pellets stained for EGFR following siRNA depletion. **C**, immunofluorescent staining of HPV⁻ HNSCC samples; representative high and low staining immunofluorescent microscopy images for each marker are shown. LC, loading control (β -actin); DAPI, nuclear stain; V, vehicle control; C, control siRNA (siGL2); IB, immunoblotting; CK, cytokeratin (epithelial tumor stain); DNA, DAPI stain. Scale bar, 100 μ m.

Figure 3.

Kaplan–Meier survival analysis for patients with high or low expression levels of EGFR, HER2, and NSDHL. **A**, Kaplan–Meier survival curves for patients in the FCCC cohort (patients treated with surgery only and patients treated with surgery and radiotherapy were included) and multivariate analysis (adjusted survival analysis; Supplementary Table S3) for the FCCC cohort. **B**, Kaplan–Meier survival curves for TCGA validation cohort based on EGFR and HER2 reverse-phase protein array data for 186 HPV[−] HNSCC samples downloaded from <https://tcga-data.nci.nih.gov/>. **C**, Kaplan–Meier survival curves based on EGFR and HER2 expression in high and low T-stage tumors, respectively. **D**, Kaplan–Meier survival curves based on NSDHL expression in high T-stage tumors (FCCC cohort). **E**, survival based on low mRNA expression (TCGA cohort) of one of the four members of the NSDHL complex (NSDHL, MSMO1, HSD17B7, and C14ORF1). **F**, mRNA expression correlation for the four genes of the NSDHL complex with statistical values. CI, confidence interval. See Supplementary Table S3 for additional details regarding the HR and Supplementary Table S1 for TCGA data.

Figure 4. Correlations between EGFR and RB1 and prognostic value. **A**, statistically significant correlations between marker expression levels (increasing saturation of blue indicates higher correlation and of red indicates inverse correlation; correlations with $P > 0.05$ are suppressed). **B**, correlation between EGFR and CCND1-normalized protein expression (Norm. protein; based on TCGA RPPA data; Supplementary Table S1); inserted violin plots indicate distribution of data points for EGFR (red) and CCND1 (blue). **C**, alterations in the indicated genes (each column represents an individual sample) and significant co-occurrence of alterations are presented [TCGA cohort; cBioportal (24) was used to generate graphs and calculate significance of co-occurrence]. **D** and **E**, Kaplan–Meier survival curves based on mRNA expression levels of EGFR and CCND1 (**D**) and EGFR and p16 (**E**). **F**, correlation between CCND1 mRNA expression (normalized TCGA z-scores) and cases with low mRNA expression of CDK6 [CDK6 (low)]. **G**, Kaplan–Meier survival curves for cases with

high mRNA expression of EGFR stratified by CDK6 expression (H, high CDK6; L, low CDK6/compensatory CCND1; M, medium CDK6/no compensatory CCND1). Medium CDK6 mRNA expression was defined as normalized TCGA z-scores between 0 to 1; high and low were defined as z-scores >1 and <0 , respectively. m , slope. mRNA data for 243 HPV⁻ HNSCC samples (6) were downloaded using cBioportal (24).

Figure 5. Targeted inhibition of EGFR and CDK4/6. **A** and **B**, cell titer blue viability assays for lapatinib (Lap), afatinib (Afa), palbociclib (Pal), and combination treatment (Combo). Coefficient of interaction values at different effective doses for the combination treatment were calculated using the Chou–Talalay method. Graphs are representative of at least two independent experiments. **C** and **D**, expression levels of the indicated proteins in FaDu (**C**) or SCC61 cells (**D**) after treatment with 0.5 $\mu\text{mol/L}$ of lapatinib (Lap.) and/or 0.5 $\mu\text{mol/L}$ of palbociclib (Pal.) for the indicated time; images are representative of at least two independent experiments. **E**, schematic representation of the proteins highlighted in **C** and **D** and related proteins. ED, effective dose; a coefficient of interaction value of >1 indicates

antagonism, = 1 indicates an additive effect, and < 1.0 indicates synergy; a coefficient of interaction < 0.5 indicates strong synergy.

Table 1

Patient characteristics

Characteristics	N (%)
Surgery	
No	28 (28%)
Yes	71 (72%)
Gender	
Female	37 (37%)
Male	62 (63%)
Age	
Mean	63.2
Min	25
Max	86
SD	12.7
Tumor site	
Glottis	16 (16%)
Hypopharynx	3 (3%)
Oral cavity	43 (43%)
Oral tongue	21 (21%)
Oropharynx	11 (11%)
Other	5 (5%)
Stage	
1	17 (17%)
2	8 (8%)
3	14 (14%)
4	35 (35%)
4A	25 (25%)
T-stage	
1	21 (21%)
1A	1 (1%)
2	25 (25%)
3	15 (15%)
3B	1 (1%)
4	34 (34%)
4A	1 (1%)
4B	1 (1%)
N-stage	
0	48 (48%)
1	12 (12%)
2	2 (2%)
2A	3 (3%)
2B	29 (29%)

Characteristics	N (%)
2C	5 (5%)
M-stage	
1	98 (99%)
2	1 (1%)
Grade	
Moderately Diff.	58 (59%)
Poor/Undiff.	30 (30%)
Well diff.	10 (10%)
N/A	1 (1%)
Alcohol	
Current	7 (7%)
Never	12 (12%)
Past	4 (4%)
Unknown	76 (77%)
Smoking	
Current	32 (32%)
Never	13 (13%)
Past	34 (34%)
Unknown	20 (20%)
Patients per marker	
EGFR	96 (97%)
HER2	92 (93%)
PTEN	91 (92%)
NSDHL	88 (89%)
BCAR1	80 (81%)

Abbreviations: %, percentage out of 99 patients; Diff., differentiated; max, maximum; min, minimum; N, number of specimens; Undiff., undifferentiated.