Correlation of Ki-67 Proliferative Antigen Expression and Tumor Response to Induction Chemotherapy Containing Cell Cycle-Specific Agents in Head and Neck Squamous Cell Carcinoma

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Abstract Determine if highly proliferative head and neck squamous cell carcinomas, assessed by pretreatment Ki-67 expression, respond more robustly to induction chemotherapy (IC) that is selectively toxic to cycling cells. Retrospective analysis of 59 patients treated with IC and chemoradiation. IC included either *nab*-paclitaxel, cisplatin, 5-FU and cetuximab (APF-C, *n*=27) or docetaxel, cisplatin, 5-FU +/- cetuximab (TPF+/-C, *n*=32). Ki-67 expression was assessed by immunohistochemistry. Tumor response (complete/partial/stable/progressive) at the primary site after two IC cycles was evaluated by visual examination in all

patients. In the APF-C sub-group, tumor response (primary site and neck nodes) after two IC cycles was evaluated by computed tomography (CT) and fluorodeoxyglucose-positron emission tomography (FDG-PET)/CT. Ki-67 expression (median 66%, range: 16–97) did not differ across the tumor response categories assessed by visual examination (*p*=0.95), CT (*p*=0.30), or FDG-PET/CT (*p*=0.65). Median decrease in summed SUV_{max} of measured lesions was 71.6% (range: 8.3–100%). The Pearson correlation coefficient between Ki-67 expression and the percent decrease in summed SUV_{max} was 0.48 (*p*=0.02). Ki-67 expression was not different between those with or without a relapse (median: 60 and 71%, *p*=0.10). In multivariate regression analysis (MVA) controlling for p16 positive oropharyngeal SCC status and smoking status, Ki-67 expression was not significantly associated with tumor response by visual examination (coefficient estimate −0.002, standard error 0.010, *p*=0.84), CT (coefficient estimate −0.007, standard error 0.011, *p*=0.54), FDG-PET/CT (coefficient estimate 0.006, standard error 0.008, *p*=0.51), the percent decrease in summed SUV_{max} (coefficient estimate 0.389, standard error 0.222, *p*=0.09), or relapse events (OR=1.02(95%CI:0.99–1.05), *p*=0.28). No significant relationships were found in MVA between pretreatment Ki-67 expression and tumor response to IC or to relapse.

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Introduction

Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer [1], is a heterogeneous disease in which sub-groups are defined by clinical criteria

(sub-site and TNM classifications) [2], genomics [3], and etiology. The current prognostic paradigm broadly divides HNSCC into human papillomavirus (HPV)-related oropharyngeal (OP) or smoking-induced, HPV-unrelated subtypes. HPV-related OP SCC has a more favorable prognosis compared to conventional, largely smoking-induced, HPV-unrelated HNSCC [4]. Other than HPV, with p16 expression as a surrogate marker, no biomarker has been validated as an independent prognostic factor in HNSCC.

In contrast to prognostic factors, predictive factors associate with treatment response. Predictive biomarkers are widely applied in many cancers to assist in making treatment decisions. Examples include *her-2-neu* expression and anti-*her-2-neu* monoclonal antibody therapy in breast [5, 6] and gastric cancer [7, 8], estrogen receptor expression and hormone therapy in breast cancer [9], activating mutations of the epidermal growth factor receptor (EGFR) and EGFR tyrosine kinase inhibitors in lung cancer [10], and activating mutations of K-RAS and EGFR monoclonal antibodies in colorectal cancer [11, 12].

In HNSCC, there are no validated predictive biomarkers for treatment effect. Although EGFR signaling is an important driver in HNSCC as demonstrated by the improvement of overall survival (OS) with the addition of cetuximab to radiation and to chemotherapy [13, 14], there are no biomarkers that predict the efficacy of EGFR inhibitors such as cetuximab [15].

The Ki-67 protein is closely linked with cellular proliferation, being present during all active phases of the cell cycle and absent in resting cells [16]. Ki-67 is a measure of the proliferative potential of several cancers, including HNSCC [17]. Ki-67 protein expression is not used as prognostic or predictive factors in HNSCC because studies evaluating Ki-67 for these purposes have been inconclusive [17–22]. However, in breast cancer, Ki-67 expression is significantly associated with a robust tumor response to hormone therapy and anthracycline-based neoadjuvant chemotherapy [23, 24]. Further, Ki-67 is a component of two commercial tests for breast cancer recurrence risk that predict efficacy of therapy [25, 26].

Ki-67 expression has not been evaluated as a predictive biomarker of tumor response to chemotherapy in HNSCC. In this retrospective analysis, we assessed Ki-67 protein expression level as a predictive biomarker of tumor response to two cycles of induction chemotherapy with *nab*-paclitaxel or docetaxel, each given with cisplatin, 5-FU, +/- cetuximab in patients with locally advanced HNSCC. Based on the strong association between high Ki-67 expression and cell proliferation, we hypothesized that tumors with higher versus lower Ki-67 expression would respond more robustly to induction chemotherapy regimens containing agents that were selectively toxic to cycling cells, including microtubule inhibitors

(*nab*-paclitaxel or docetaxel), antimetabolites (5-FU), and an EGFR inhibitor (cetuximab) [27, 28].

Patients and Methods

Patient Selection Criteria

This retrospective protocol was reviewed and approved by the institutional review board (IRB). Eligible patients had HNSCC treated with induction chemotherapy and chemoradiation (CRT) administered at Washington University in St. Louis between 2006 and 2014. Participation in an IRB-approved tumor registry protocol was required. Eligible patients had locally advanced (Stages III-IVa/b) mucosal SCC of the oropharynx, larynx, or hypopharynx. Patients were required to be at least 18 years of age and have adequate Eastern Cooperative Group (ECOG) performance status (0–2) and vital organ function to undergo induction chemotherapy and CRT. Induction chemotherapy must have included either *nab*-paclitaxel, cisplatin, 5-fluorouracil, and cetuximab (APF-C) or docetaxel, cisplatin, 5-fluorouracil without or with cetuximab (TPF or TPF+C). CRT included single agent cisplatin, cetuximab, carboplatin, or docetaxel given concurrently with definitive intensity modulated RT. Patients scheduled to receive CRT but who only received RT because of interim development of absolute contraindications to cisplatin or cetuximab (inadequate renal function or hypersensitivity reaction [HSR], respectively) were eligible. Archived tumor tissue, formalin fixed, paraffin-embedded, pretreatment biopsy specimens for biomarker assessment were required.

Study Design and Definitions of Tumor Response to Induction Chemotherapy

Patients given APF-C and CRT were treated on a previously reported prospective phase II trial and the outcomes were updated for this analysis [4]. Patients given TPF+/-C and CRT were treated as standard of care. Induction chemotherapy consisted of every 3 week cycles of APF-C or TPF+/-C. Doses, schedules, and dose modifications of each regimen were previously reported [29].

After two cycles of induction chemotherapy, an assessment of tumor response at the primary site (defined as tumor response by visual examination) was performed in all patients by clinical examination (laryngoscopy in office or operating room) by experienced oncologic surgeons, using categorical outcomes commonly employed by others with slight modifications [30, 31]: complete response (CR), defined as complete resolution of lesion or near CR, with minimal residual mucosal abnormality; partial response (PR), defined as 50–94% decrease; stable disease

(SD), defined as 0–49% decrease; and progressive disease (PD), defined as any increase, as previously described [4]. Patients with favorable (CR, PR) tumor response at the primary site received a third cycle of induction chemotherapy before definitive CRT whereas patients with an unfavorable (SD, PD) tumor response at the primary site proceeded to definitive CRT.

Computed tomography (CT) scan of the neck and F-18 fluorodeoxyglucose-positron emission tomography (FDG-PET)/CT was performed at baseline and after two cycles of induction chemotherapy in patients given APF-C, but not in patients given TPF+/-C. RECIST version 1.0 was used to determine tumor response (at the primary tumor site and neck nodes, collectively defined as overall tumor response) by CT [32]. Tumor response as evaluated by CT (defined as the categorically assessed CT response) was categorized as CR, PR, SD or PD. Metabolic tumor response (overall) evaluated by FDG-PET/CT (defined as the categorically assessed FDG-PET/CT response) was categorized as complete metabolic response (CMR), partial metabolic response (PMR), stable metabolic disease (SMD), or progressive metabolic disease (PMD) [33]. Also, one to three tumor lesions were measured individually before and after two cycles of APF-C and the percent decrease in the total SUV_{max} of the measured lesion(s) was calculated for each patient (defined as the percent decrease in summed SUV_{max}).

Definitive CRT began 22–56 days after initiation of cycle 3 of induction chemotherapy, and was given as previously described [4]. Patients with serum creatinine <2.0 mg/dL were scheduled to receive high-dose bolus cisplatin (100 mg/m² on days 1, 22 and 43) with RT. Patients who failed to meet these criteria and who had no serious HSR with prior cetuximab received single-agent cetuximab concurrently with RT. Patients who failed to meet criteria for either cisplatin or cetuximab received RT alone. Alternatively, some patients were given carboplatin or docetaxel concurrently with RT, at physician discretion.

Standard Assessments

Baseline assessments included history and physical examination, laryngoscopy, and CT of the neck. After completion of all therapy, patients were assessed for tumor response with physical examination and laryngoscopy every 3–6 months and CT of the neck and chest performed at 6, 12, 20, 28 and 36 months. Smokers were defined as current or former tobacco users.

Studies Performed on Archived Tumor Tissue

Ki-67 expression was assessed by immunohistochemistry (IHC) on archival formalin-fixed, paraffin-embedded

(FFPE) tumor tissue obtained at diagnosis. In brief, a subspecialty head and neck surgical pathologist (JSL) utilized a Ventana Benchmark autostainer and a commercially available antibody (Ventana, Inc., clone 30–9; catalog #790-4286; prediluted) to stain tumor specimens. The specimens were digitally scanned on an Aperio Scanscope at 400x magnification, and the Aperio Nuclear v9 pre-written algorithm was used to count the percentage of tumor nuclei staining positive in manually selected tumor regions to exclude intervening stromal and inflammatory cells. The positive tumor nuclei included those of all intensity (1+, 2+, 3+) versus those read as negative (0). Archival FFPE tumor tissue was also used to assess p16 status, a surrogate marker of HPV infection, for oropharyngeal SCC patients where this was not already performed as part of the initial diagnostic workup. Methodology for p16 by immunohistochemistry was previously described [34]. Patients with greater than 50% nuclear and cytoplasmic staining were considered positive.

Hypothesis, Objectives, and Statistical Analyses

We hypothesized that expression levels of Ki-67 would be significantly correlated with a favorable response to two cycles of induction chemotherapy. The primary efficacy endpoint, assessed in all patients, was tumor response by visual examination. Secondary efficacy endpoints, assessed only in patients given APF-C, included categorically assessed CT and FDG-PET/CT response as well as the percent decrease in summed SUV_{max} . The endpoint of tumor relapse events was assessed in all patients.

In the univariate analysis, Ki-67 protein expression was compared by a Kruskal–Wallis test for primary outcome, CT and FDG-PET/CT response, and tumor relapse events. The Pearson correlation between Ki-67 protein expression and the percent decrease in summed SUV_{max} was computed. We included Ki-67 protein expression, p16 positive oropharyngeal SCC status, and smoking status in our multivariate analyses (MVA). Linear regression models were considered for tumor response by visual examination, CT and FDG-PET/CT response, and the percent decrease in summed SUV_{max} . A logistic regression was used to analyze the relationship between Ki-67 protein expression and tumor relapse events. All analyses were carried out using SAS.

Results

Patient, Tumor, and Treatment Characteristics

Fifty-nine patients met all eligibility criteria and were the subjects of this analysis. Forty-three other patients (42

treated with TPF+/-C and one treated with APF-C) were ineligible because of lack of available or adequate archival tumor tissue for evaluation of Ki-67 expression. Patient, tumor, and treatment characteristics for the 59 eligible patients are described in Table 1. Most patients were smoking males with significant co-morbidities. Median age was 55 years (range: 19–71). The most common primary tumor sites were oropharynx (71%) and larynx (25%). Twenty-eight patients (47%) had p16 positive (HPV-related) oropharyngeal SCC and 31 patients (53%) had HPV-unrelated HNSCC.

The majority of cancers were stages IVa and IVb (85%). The distributions of induction chemotherapy regimens given to patients were 46% APF-C, 46% TPF+C, and 8% TPF. Fifty-one patients (86%) were treated with CRT and two patients (3%) were treated with RT alone.

Table 1 Patient, tumor and treatment characteristics

Characteristic	Number	Percentage (%)
Patient		
Age		
Median (range)	55 (19–71)	
Sex		
Male	54	92
Female	5	8
Smoking		
Yes	53	90
No	6	10
ACE comorbidity index		
0	15	25
1	19	32
2	15	25
3	10	17
Tumor		
Primary site		
Oropharynx	42	71
p-16 pos	28	47
p-16 neg	14	24
Larynx	15	25
Hypopharynx and oral cavity	2	3
Stage		
III	9	15
IVA	36	61
IVB	14	24
Treatment		
APV-C	27	46
TPF-C	27	46
TPF	5	8

Six patients (10%) were not treated with RT because of death (2), disease progression (2), redirection to surgery (1), and loss to follow-up (1). Of the 51 patients treated with CRT, 41 (80%) received concurrent cisplatin, six (12%) cetuximab, three (6%) carboplatin, and one (2%) docetaxel.

Ki-67 Protein Expression by Tumor Site, Stage and Induction Chemotherapy Regimen

The distribution of Ki-67 protein expression levels based on primary tumor site, stage, and induction chemotherapy regimen is described in Fig. 1 and Table 2. Median

Fig. 1 Distribution of Ki-67 protein expression in the pretreatment tumor specimens across the 59 study patients

Table 2 Ki-67 Expression by subgroup

Characteristic	Number	%	Median Ki-67 expression % (range)
Primary site			
Oropharynx	42	71	71 (23–97)
p-16 pos	28	47	76 (45–97)
p-16 neg	14	24	62 (23–86)
Larynx	15	25	54 (16–90)
Hypopharynx and oral cavity	2	3	47 (35–60)
Stage			
III	9	15	59 (30–90)
IVA	36	61	65 (16–97)
IVB	14	24	69 (34–95)
Treatment			
APV-C	27	46	67 (23–97)
TPF-C	27	46	60 (16–94)
TPF	5	8	64 (30–76)

Ki-67 protein expression was 66% (range: 16–97%). Ki-67 expression was higher in oropharyngeal SCC than in laryngeal SCC ($p=0.0037$). A comparison to hypopharynx and oral cavity SCC was not performed because of the limited number of cases. Ki-67 expression level did not differ across patients with different stages of disease ($p=0.26$) or across the three induction chemotherapy regimen groups ($p=0.40$).

Ki-67 Expression and Tumor Response to Induction Chemotherapy

Ki-67 protein expression in each of the tumor response assessment categories is detailed in Table 3. Tumor response by visual examination was (Table 4) evaluable in 58 of the 59 patients: CR occurred in 25 (43%), PR in 28 (48%), and stable disease or progressive disease in five patients (9%). Of those treated with APF-C, CR occurred in 15 and PR occurred in 12 patients. Of those treated with TPF+/-C, CR occurred in 10, PR in 16, and stable disease

Table 3 Ki-67 Expression by response to chemotherapy

Characteristic	Number	%	Median Ki-67 expression % (range)	p value
Tumor response by visual exam				0.95
CR	25	45	68 (37–95)	
PR	28	46	67 (16–97)	
SD	1	2	60 (n/a)	
PD	4	7	64 (59–84)	
Categorical CT response				0.30
CR	4	13	47 (31–89)	
PR	13	50	80 (45–94)	
SD	8	33	72 (23–97)	
Prog	1	4	62 (n/a)	
Categorical FDG-PET/CT response				0.65
CMR	6	24	81 (51–91)	
PMR	17	64	66 (34–95)	
SMD	1	4	23 (n/a)	
PMD	2	8	79 (62–97)	
Percent decrease in summed SUV _{max}				0.02
Decrease $\geq$ median	12	50	72 (43–95)	
Decrease < median	12	50	66 (23–97)	
Relapse				0.10
No relapse	40	67	71 (16–97)	
Relapse	19	33	60 (23–84)	

CR complete response; PR partial response; SD stable disease; PD progressive disease

CMR complete metabolic response; PMR partial metabolic response; SMD stable metabolic disease; PMD progressive metabolic disease

Table 4 Association of Ki-67 staining area with the response to induction chemotherapy and relapse

Dependent variable	Coefficient	Standard error	p value
Tumor response by visual exam ^a	−0.0020	0.010	0.84
Categorically assessed CT response ^a	−0.007	0.011	0.54
Categorically assessed FDG-PET/CT response ^a	0.006	0.008	0.51
Percent decrease in summed SUV _{max}	0.389	0.222	0.09
	Odds ratio	95% CI	
Relapse	1.02 ^b	0.99–1.05	0.28

Ki-67 is analyzed as a continuous variable

^aA higher value corresponds to a more favorable categorical response

^bOdds of remaining relapse-free

or progressive disease in five patients. Ki-67 expression level did not significantly differ across the categories of tumor response by visual examination ($p=0.95$).

In patients treated with APF-C, categorically assessed CT response was evaluable in 26 of the 27 patients: CR occurred in 4 (15%), PR in 13 (50%), and SD or progressive disease in nine patients (35%). Ki-67 expression level was not significantly different across these categories of assessed CT response ($p=0.30$). In the patients treated with APF-C, categorically assessed FDG-PET/CT response was evaluable in 26 of the 27 patients: CMR occurred in 6 (23%), PMR in 17 (65%), SMD or progressive disease in three patients (12%). Ki-67 expression level was not significantly different across these categories assessed by FDG-PET/CT response ($p=0.65$). The percent decrease in summed SUV_{max} of measured lesions was evaluable in 24 patients. The median decrease in summed SUV_{max} was 71.6% (range: 8.3–100%). The Pearson correlation coefficient between Ki-67 expression level and the percent decrease in summed SUV_{max} was 0.48 ($p=0.02$).

Ki-67 Expression and Tumor Relapse

At a median follow-up of 6.5 years (range: 5.4 to 10.0 years) for all surviving patients, disease relapse occurred in 19 (33%) patients. Ki-67 protein expression level was not significantly different between those with and without relapse events (Table 3). Median Ki-67 expression levels were not significantly different between patients with relapse events (60%; range: 23–84%) compared to those without relapse events (71%; range: 16 to 97%) ($p=0.10$).

Association of Tumor Response to Induction Chemotherapy and Relapse to Ki-67 Expression

In a multivariate regression analysis controlling for p16 positive oropharyngeal SCC status and smoking status, Ki-67 expression was not significantly associated with tumor response as assessed by visual examination (coefficient estimate -0.002 , standard error 0.010, $p=0.84$), CT (coefficient estimate -0.007 , standard error 0.011, $p=0.54$), FDG-PET/CT (coefficient estimate 0.006, standard error 0.008, $p=0.51$), the percent decrease in summed SUV_{max} (coefficient estimate 0.389, standard error 0.222, $p=0.09$), or relapse events (OR = 1.02 (95% CI: 0.99–1.05), $p=0.28$).

Discussion

This report is the first to evaluate Ki-67 protein expression as a potential predictive biomarker of tumor response to chemotherapy in HNSCC. We hypothesized that highly

proliferative tumors, assessed by the surrogate marker of Ki-67 protein expression, would respond more robustly to induction chemotherapy agents that were selectively more toxic to cycling cells, including microtubule inhibitors, antimetabolites, and an EGFR inhibitor [27, 28]. In multivariate regression analysis, we did not find a significant relationship between pretreatment Ki-67 protein expression level and tumor response to the induction chemotherapy regimens of APF-C and TPF+/-C as assessed by visual examination of the primary site, anatomic change based on CT scan, or metabolic change based on FDG-PET/CT. We also looked at relapse events, and found no significant association between Ki-67 expression level and this important long term clinical endpoint.

In controlled trials of endocrine therapy for estrogen receptor-positive breast cancer, dynamic changes in Ki-67 expression levels measured 2 weeks after starting therapy, as opposed to static pretreatment Ki-67 expression, were associated with recurrence-free survival [24, 35]. Greater reductions in Ki-67 expression with endocrine therapy associated with more benefit. This observation is thought to occur because the benefits of endocrine therapy in breast cancer are related to its ability to inhibit estrogen-induced cellular proliferation, which is not directly related to pretreatment Ki-67 expression level [36]. If Ki-67 protein expression and tumor response to chemotherapy were similarly associated in HNSCC, the design of our study would not have detected this dynamic relationship since we only assessed pretreatment Ki-67 expression.

In our study, there are other potential explanations for the lack of association between baseline Ki-67 expression level and tumor response to induction chemotherapy. First, the limited number of patients lowered the power of the analysis to identify small differences in outcomes between groups with higher and lower expression levels of Ki-67. Second, heterogeneity in patient and tumor characteristics may not have effectively been controlled for as prognostic variables in the analysis. Third, technical variability in Ki-67 assessment by IHC may limit the precision and accuracy of the measurement. Fourth, the majority of patients achieved a complete or partial categorical tumor response to induction chemotherapy, which may have lowered the power of the analysis to detect differences across Ki-67 expression levels. Fifth, Ki-67 protein expression may be a suboptimal surrogate biomarker of cellular proliferation. Finally, the null hypothesis may be true, that is, baseline Ki-67 expression level may not be a predictive biomarker for tumor response of HNSCC to induction chemotherapy agents that are selectively more toxic to cycling cells.

Although the results of this retrospective analysis failed to show an association between Ki-67 expression level and tumor response to induction chemotherapy in locally advanced HNSCC, these data can be used as a benchmark

to design prospective trials evaluating this relationship. Prospective trials can reduce the impact or eliminate the limitations of this retrospective analysis. Although the clinical application of the hypothesis of our retrospective analysis may be critiqued due to the decline in interest in induction chemotherapy in HNSCC following two negative randomized induction chemotherapy trials [37, 38], chemotherapy remains the backbone of treatment of incurable HNSCC and in this setting the potential of Ki-67 expression as a predictive marker of tumor response to chemotherapy should be assessed. Our analysis provides benchmark data to develop prospective trials to further assess this question.

In conclusion, our exploratory study does not support the clinical use of pretreatment levels of Ki-67 protein expression as a predictive biomarker of tumor response to induction chemotherapy in patients with HNSCC. Further work should be done to investigate whether post-treatment changes in Ki-67 expression or if other biomarkers associate with tumor response to chemotherapy in patients with HNSCC.

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Compliance with Ethical Standards

Conflict of interest Dr. Adkins has received Research Funding from Celgene. All other authors have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study, as part of a registry protocol.

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