Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Bcl2 and Human Papilloma Virus 16 as Predictors of Outcome following Concurrent Chemoradiation for Advanced Oropharyngeal Cancer

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Abstract

Purpose: Oropharyngeal squamous cell carcinoma (OPSCC) associated with human papilloma virus (HPV) is rapidly growing in incidence. Despite better prognosis than OPSCC associated with traditional risk factors, treatment failure still occurs in a significant proportion of patients. We had identified the antiapoptotic protein Bcl2 as a marker for poor outcome in advanced OPSCC treated with concurrent chemoradiation. To determine whether Bcl2 and HPV together might further characterize treatment response, we examined whether the prognostic value of Bcl2 was independent of HPV status.

Experimental Design: Pretreatment tumor biopsies from 68 OPSCC patients were tested for HPV by *in situ* hybridization and were immunostained for Bcl2 to evaluate relations with disease-free (DFS) and overall survival following platin-based concurrent chemoradiation. Median follow-up among surviving patients was 47 months (range, 10-131 months).

Results: Bcl2 and HPV independently predicted DFS and overall survival. Hazard ratios (with 95% confidence interval) for positive versus negative status in bivariate Cox proportional hazard analysis of DFS were 6.1 (1.8-21) for Bcl2 and 0.11 (0.035-0.37) for HPV. Only 1 of 32 HPV-positive/Bcl2-negative tumors recurred. Pretreatment Bcl2 expression was specifically associated with distant metastasis; five of six distant metastases occurred in the <40% of patients whose primary tumors were Bcl2 positive.

Conclusions: Independent of HPV status, pretreatment Bcl2 expression identifies a subset of OPSCC patients having increased risk of treatment failure, particularly through distant metastasis, after concurrent chemoradiation. Considering HPV and Bcl2 together should help in devising better personalized treatments for OPSCC. *Clin Cancer Res; 16(7); 2138–46. ©2010 AACR.*

Patients with human papillomavirus (HPV)–related tumors represent an increasing fraction of newly diagnosed squamous cell carcinomas of the head and neck (HNSCC; refs. 1, 2). These tumors, almost solely oropharyngeal squamous cell carcinomas (OPSCC), typically present at an advanced stage by traditional tumor-node-metastasis (TNM) criteria, yet respond better to treatment than do tumors associated with traditional risk factors (3–11). Nevertheless, a significant fraction of HPV-related tumors resist current standards of care, so it is important to find additional ways to determine prognosis and guide therapy.

Cellular mechanisms inhibiting apoptosis can contribute to poor treatment outcome. In HNSCC cell lines, we found that high expression of the antiapoptotic protein Bcl2 enhanced tumor-cell survival, in particular after treatment with cisplatin (12, 13). We extended these findings to advanced OPSCC treated with platinum-based concurrent chemoradiation, a current standard of care (9, 14, 15), and determined that high pretreatment tumor expression of Bcl2 predicted worse clinical outcome (13).

These findings suggest that considering HPV and Bcl2 together might further classify OPSCC with respect to therapeutic response. To evaluate this possibility, it is crucial to determine whether Bcl2 and HPV are independently related to outcome. Statistical power to resolve this issue required larger numbers of patients than examined in our earlier studies. We now report in this larger study that HPV infection and low Bcl2 expression are independent predictors of improved outcome in OPSCC treated with concurrent chemoradiation and that high pretreatment

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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-09-3185

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Translational Relevance

As oropharyngeal squamous cell carcinoma (OPSCC) associated with human papilloma virus (HPV) grows rapidly in incidence, better ways are needed to determine prognosis and to individualize treatment. We had previously identified the antiapoptotic protein Bcl2 as a marker for poor outcome in advanced OPSCC treated with concurrent chemoradiation. We now find that this prognostic significance of Bcl2 is independent of HPV status, so that the combination of HPV and Bcl2 pretreatment status provides better prognostic information than either pretreatment status individually. In particular, almost all patients with HPV-positive/Bcl2-negative OPSCC were cured by concurrent chemoradiation, identifying a subset of patients for whom less aggressive treatments might be considered. Bcl2-positive tumors were specifically associated with treatment failure through distant metastases, suggesting that more aggressive systemic chemotherapy might be required for patients having such tumors.

Bcl2 is specifically associated with failure through distant metastasis.

Materials and Methods

Patient inclusion criteria and treatment. With permission from the local Institutional Review Board for retrospective case review, in accordance with an assurance filed with and approved by the U.S. Department of Health and Human Services, we identified 68 patients, treated in the Partners Healthcare System, having: (*a*) biopsy-proven squamous cell carcinoma of the oropharynx, (*b*) no prior HNSCC, (*c*) no prior head and neck irradiation, (*d*) pretreatment biopsy paraffin block available, (*e*) follow-up for at least 2 y or until death or a recurrence proven by biopsy, and (*f*) definitive concurrent chemoradiation treatment, with or without neck dissection, with a platinum-based chemotherapeutic agent. Of 235 OPSCC patients, 71 had treatments other than concurrent platinum-based chemoradiation and pathology blocks were unavailable for another 96. The remaining 68 patients reported here included 38 evaluated in a previous study (13). Entry into the study was set as the initial biopsy date (between May 1996 and February 2006). TNM staging followed standard criteria (9). Patients were identified as smokers if they reported 1 pack-year or more history of smoking. Alcohol use was scored as "excessive" for patients who reported a history of more than five drinks per day or had the terms "alcoholism," "alcohol abuse," "alcohol dependence," or "alcoholic" noted in medical records.

Radiotherapy was delivered, generally as intensity-modulated radiation therapy five times weekly in daily fractions of 2 to 2.1 Gy for a total of 33 to 40 fractions. Concurrent chemotherapy was administered either in a high-dose (cisplatin: 100 mg/m^2 i.v. over 1 h every 3 wk for up to three cycles, 12 patients) or in a weekly regimen [carboplatin: area under curve (AUC), 1.5 i.v. over 30 min, 56 patients; typically plus paclitaxel, 45 mg/m^2 i.v. over 30 min, up to seven treatments, 49 patients] as tolerated. One patient receiving cisplatin also received paclitaxel.

Patients were evaluated closely at 4- to 6-wk intervals during the first 2 y of follow-up. The median follow-up time after initial biopsy among surviving patients was 47 mo (range, 10-131 mo). Disease-free survival (DFS) was defined as the time from pretreatment biopsy to local, regional, or distant recurrence; overall survival (OS) was defined as the time from pretreatment biopsy to death from any cause.

IHC and* in situ *hybridization. Formalin-fixed, paraffin-embedded specimens from pretreatment biopsies were sectioned at 5 μm. A study pathologist (WCF) confirmed the presence of tumor in sections stained with H&E. Further sections were then processed with *in situ* hybridization (ISH) for HPV and with immunohistochemistry (IHC) for Bcl2, BclX_L, and the HPV surrogate p16 protein (16), following previously described standardized techniques (11, 13). Results were scored by pathologists blinded to patient outcomes.

HPV* in situ *hybridization. HPV16 DNA (the most prevalent HPV type in OPSCC) was detected with the ISH-catalyzed signal amplification method for biotinylated probes (DAKO GenPoint; ref. 17). HPV16-positive controls included an HPV16-positive OPSCC, the SiHa cell line (one to two integrated copies of HPV16), and the CaSki cell line (~500 integrated copies). Punctuate hybridization signals localized to the tumor cell nuclei defined an HPV-positive tumor (18, 19). Tumors p16 positive by IHC but HPV16 negative by ISH were further evaluated for 12 additional oncogenic HPV types (types 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) using a biotinylated probe cocktail (GenPoint HPV Probe Cocktail, DAKO).

Bcl2 and BclX_L IHC. Endogenous peroxidase was blocked by H_2O_2 and antigens were retrieved with EDTA plus boric acid in Tris buffer (CC1 reagent; Ventana) for 30 to 60 min. Primary antibodies were Bcl2 prediluted antibody (Ventana 760-4240; 1 h, 52 min) and BclX_L (1:40 dilution; NeoMarkers MS-1334-P1; 32 min). Secondary antibodies were UltraView horseradish peroxidase–conjugated multimers; detection was with UltraView diaminobenzidine chromogen (Ventana). Tissues were counter-stained with hematoxylin. The criteria of Jäckel et al. (20) were used for Bcl2 and BclX_L. An intensity score (0, absent; 1, weak; 2, moderate; 3, strong) and a prevalence score (0, <25% of tumor stained; 1, 25-75% stained; 2, >75% stained) were added; a total of three or greater was called positive. Stromal lymphocytes in tonsil provided positive controls for Bcl2 and BclX_L staining.

p16 IHC. The CDK-inhibitor p16, a biomarker of HPV E7 oncoprotein activity (21–24), was detected in deparaffinized

Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

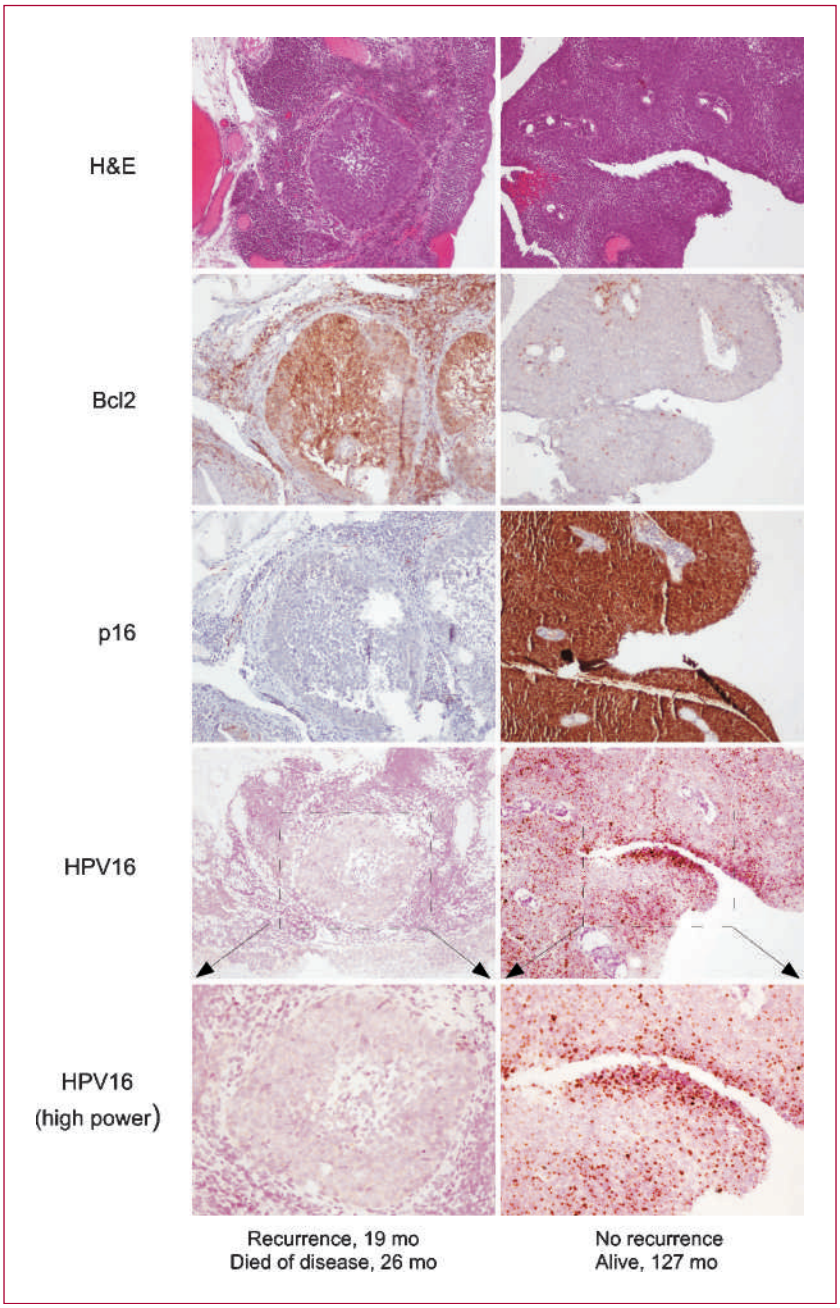

Fig. 1. Staining for Bcl2 (IHC), p16 (IHC), and HPV16 (ISH) as described in the text. Each column shows sections of pretreatment biopsies from a single patient whose outcome is indicated. Classifications: left, Bcl2 positive, p16 negative, HPV16 negative; right, Bcl2 negative, p16 positive, HPV16 positive. High-power HPV16-positive image shows punctuate staining.

sections subjected to antigen retrieval using 10 mmol/L citrate buffer (92°C for 30 min). A primary mouse monoclonal antibody against p16 (MTM Laboratories) was visualized with the Ventana XT autostainer using the one-view secondary detection kit (Ventana). p16 expression was scored as positive if strong and diffuse nuclear and cytoplasmic staining was present in ≥70% of the tumor specimen. Cervical carcinoma provided a positive control for p16 staining.

Figure 1 shows representative photomicrographs of sections of tumors from two patients, with IHC for Bcl2 and p16, and with ISH for HPV16.

Statistical methods. The Cox proportional hazard model was fit to estimate the relative hazard of mortality or recurrence. In addition to univariate analyses based on each patient characteristic, we performed bivariate analyses specifying Bcl2 and HPV as factors to test our hypothesis that the combination of these markers would predict outcome. Due to the limited sample size, we performed confirmatory stratified exact log-rank tests (Statexact V6, Cytel Software Corp) to evaluate independent influences of Bcl2 and HPV on recurrence. To determine whether additional variables might help predict outcome, we also performed

Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

multivariate analyses of DFS and OS with forward stepwise Cox regression that considered all variables, retaining those in the results of the stepwise analyses that had *P* values of 0.05 or less. A two-sided *P* value of 0.05 or less was considered statistically significant.

Results

Bcl2 was unrelated to HPV status or to other patient characteristics. Of 68 patients, 53 (78%) had HPV16-positive tumors, whereas 56 (82%) and 24 (35%) tumors expressed p16 and Bcl2, respectively. No HPV type other than HPV16 was detected in p16-positive tumors. Detailed patient characteristics and marker status are presented in Table 1. HPV16 infection and p16 expression were highly associated, agreeing in 65 cases (96%; $P < 0.001$, Fisher exact test), similar to most previous reports (4, 6–8, 16, 18, 19, 21, 22, 24). All HPV16-positive tumors were positive for p16. Consistent with other reports, HPV16-related tumors were associated with smaller T stage, advanced N stage, nonsmokers, and those without excessive alcohol use. In contrast, Bcl2 was not significantly associated with any patient characteristic, HPV16 infection, or p16 expression. Because others have reported a relation of the Bcl2-related protein $BclX_L$ to outcome in HNSCC (8, 25), we also determined $BclX_L$ status and found that it was not associated with patient characteristics, HPV16, p16, or Bcl2. The high-dose and weekly chemotherapy groups did not differ significantly in terms of patient characteristics or expression of the four biomarkers we examined (data not shown).

Bcl2 and HPV together predicted outcome. Thirteen patients (19%) developed recurrence, nine of whom eventually succumbed to disease. Four patients died of other causes, with no deaths related to treatment.

Univariate analysis (Table 2) identified HPV16 infection, p16 expression, and low Bcl2 expression as predictors of

Table 1. Patient characteristics and relations to markers

Characteristic or marker		Total	HPV16			p16			Bcl2			$BclX_L$		
			–	+	*P**	–	+	*P**	–	+	*P**	–	+	*P**
Age	<60 y	44	7	37	0.13	6	38	0.32	27	17	0.60	26	18	0.80
	≥60 y	24	8	16		6	18		17	7		13	11	
Gender	Female	10	2	8	1	1	9	0.68	9	1	0.085	5	5	0.73
	Male	58	13	45		11	47		35	23		34	24	
Site	Tonsil	35	6	29	**0.048**	4	31	**0.026**	22	13	1	21	14	0.67
	Tongue base	29	6	23		5	24		19	10		15	14	
	Other	4	3	1		3	1		3	1		3	1	
T stage	1	18	1	17	**<0.001**	1	17	**0.003**	12	6	0.63	6	12	0.083
	2	33	3	30		3	30		19	14		21	12	
	3	10	6	4		5	5		8	2		8	2	
	4	7	5	2		3	4		5	2		4	3	
N stage	0	7	5	2	**0.012**	4	3	**0.049**	6	1	0.20	5	2	0.71
	1	11	2	9		2	9		8	3		5	6	
	2	46	7	39		6	40		26	20		27	19	
	3	4	1	3		0	4		4	0		2	2	
Stage	II	1	0	1	0.45	0	1	0.28	0	1	0.12	1	0	0.87
	III	15	5	10		5	10		12	3		8	7	
	IV	52	10	42		7	45		32	20		30	22	
Smoking	<1 pack-year	33	1	32	**<0.001**	1	32	**0.003**	18	15	0.13	17	16	0.46
	≥1 pack-year	35	14	21		11	24		26	9		22	13	
Excessive	No	53	6	47	**<0.001**	4	49	**<0.001**	34	19	1	29	24	0.56
Alcohol use	Yes	15	9	6		8	7		10	5		10	5	
HPV16	Negative	15				12	3	**<0.001**	12	3	0.23	9	6	1
	Positive	53				0	53		32	21		30	23	
p16	Negative	12							9	3	0.52	7	5	1
	Positive	56							35	21		32	24	
Bcl2	Negative	44										25	19	1
	Positive	24										14	10	
$BclX_L$	Negative	39												
	Positive	29												

*Fisher exact test; $P < 0.05$ highlighted in bold.

Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

Table 2. Univariate relations to DFS and OS

		DFS		OS	
		HR (95% CI)*	$P^{\dagger}$	HR (95% CI)*	$P^{\dagger}$
Age	≥60 vs <60 y	0.50 (0.14-1.81)	0.29	0.85 (0.26-2.8)	0.79
Gender	Male vs female	26.5 (0.05-10,000)	0.31	31.8 (0.06-10,000)	0.28
Site	Pharynx, palate vs base of tongue, tonsil	1.63 (0.21-12.6)	0.64	5.0 (1.5-23.7)	0.043
T stage	3,4 vs 1,2	4.36 (1.46-13.0)	0.008	2.60 (0.82-8.3)	0.11
N stage	2,3 vs 0,1	2.05 (0.45-9.2)	0.35	1.03 (0.28-3.8)	0.97
Stage	IV vs II,III	4.05 (0.53-31.1)	0.18	1.53 (0.33-7.0)	0.58
Smoking	≥1 vs <1 pack-year	2.34 (0.72-7.6)	0.16	2.03 (0.61-6.8)	0.25
Excessive alcohol use	Yes vs no	3.71 (1.24-11.1)	0.019	2.84 (0.90-9.0)	0.075
Chemotherapy	High-dose vs weekly	0.81 (0.18-3.67)	0.79	1.41 (0.38-5.3)	0.61
HPV16	Positive vs negative	0.21 (0.069-0.61)	0.005	0.40 (0.13-1.25)	0.11
p16	Positive vs negative	0.22 (0.073-0.65)	0.006	0.42 (0.12-1.4)	0.15
Bcl2	Positive vs negative	3.25 (1.06-10.0)	0.039	4.09 (1.23-13.6)	0.022
$BclX_L$	Positive vs negative	1.16 (0.39-3.45)	0.79	1.87 (0.59-5.9)	0.29

*CI by Cox proportional hazard analysis.
†Wald test.

longer DFS ($P = 0.005$, 0.006, and 0.039, respectively) and low Bcl2 as a predictor of longer OS ($P = 0.022$). The magnitudes of the hazards for DFS and for OS that were associated with high Bcl2 were over 3.0, similar magnitudes as those associated with lack of HPV. $BclX_L$ expression, in contrast, was not a statistically significant predictor of either DFS or OS. Univariate analysis also revealed clinical and behavioral characteristics associated with HPV-positive tumors (low T stage, no excessive alcohol use, primary site in tonsil or base of tongue; Table 1) to be related to better outcome, consistent with the better outcomes expected for HPV-associated OPSCC. There was no significant difference between high-dose and weekly chemotherapy in terms of DFS or OS.

Because Bcl2 status was unrelated to HPV status among these tumors, and we hypothesized that these predictors would be independently related to outcome, we evaluated these markers in combination. Figure 2 shows the relation of individual outcomes to HPV and Bcl2 status, and Fig. 3 shows Kaplan-Meier curves for DFS and OS based on combinations of HPV and Bcl2 status. Only 1 of the 32 patients whose tumors had both favorable markers (HPV16 positive and Bcl2 negative) had recurrent disease and neither of the 2 deaths among those 32 patients resulted from OPSCC. In contrast, the three patients whose tumors had neither favorable marker had recurrent disease by 26 months; one was rescued by local surgical salvage whereas 2 died of OPSCC. Patients having HPV-positive/Bcl2-positive tumors showed similar DFS and OS progressions as those with HPV-negative/Bcl2-negative tumors.

Bcl2 and HPV status each had substantial implications for outcome when the other was taken into account, as we had hypothesized. Exact log-rank tests showed a highly significant relation both of HPV to DFS after stratification by Bcl2 ($P = 0.0003$) and of Bcl2 to DFS after stratification by HPV ($P = 0.002$). To estimate the magnitudes of the hazards associated with HPV and Bcl2, we performed Cox proportional hazard analysis both for the bivariate combination of HPV with Bcl2 and for each marker stratified by the other (Table 3). In the model for DFS including both Bcl2 and HPV, the hazard ratio (HR) for each was 6.1 [95% confidence interval (95% CI), 1.8-21; $P = 0.004$] and 0.11 (95% CI, 0.035-0.37; $P < 0.001$), respectively. HRs in the model for OS were similar (Table 3).

Stepwise forward Cox analysis, allowing consideration of all variables in Table 2, identified only Bcl2 and HPV as significant predictors of either DFS or OS. Significant relations of HPV and Bcl2 to both DFS and OS were also seen when patients were stratified by chemotherapy treatment (data not shown), indicating that the relations of HPV and Bcl2 to outcome did not depend on the particular choice of platin-based chemotherapy.

We found that p16 expression could provide a proxy for HPV status, as others have suggested (6, 8, 18, 22, 24). Results from analyses based on Bcl2 and p16 were similar to those for Bcl2 and HPV (Supplementary Table S1; Supplementary Fig. S1). One of three patients with a Bcl2-negative tumor that was p16 positive but HPV16 negative, however, had recurrent disease in 7 months and died of disease at 20 months.

Bcl2 was associated with distant recurrence. Concurrent platinum-based chemoradiation for HNSCC can result in more failures through distant metastasis than found with treatments that involve more aggressive systemic therapy (26). Tumors expressing antiapoptotic Bcl2 thus might be overrepresented in distant failures after concurrent chemoradiation. Univariate Cox proportional hazard analysis supported this hypothesis, showing a HR for distant recurrence in patients having Bcl2-positive primary tumors of 10.4 (95% CI, 1.2-89; $P = 0.033$). Five of six distant recurrences

Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

by 36 months (highlighted in bold in Fig. 2) were in the <40% of patients whose primary tumors were Bcl2-positive ($P = 0.023$, Fisher's exact test, based on 22 Bcl2-positive and 37 Bcl2-negative cases followed up for at least 36 months or until recurrence). No other patient characteristics, including HPV status, showed association with distant recurrence in univariate analysis (data not shown). Importantly, Bcl2 was not significantly associated with locoregional recurrence ($P = 0.60$), suggesting that distant failure in Bcl2-positive tumors was independent of locoregional disease.

Discussion

These results show that high Bcl2 expression portends worse outcome of advanced OPSCC after concurrent platin-based chemoradiation, regardless of HPV status. The reduced hazards for DFS and OS associated with Bcl2-negative pretreatment tumors were close in magnitude to those associated with HPV infection, in univariate, bivariate, and stratified analyses (Tables 2 and 3). Treatment failure in HPV-positive/Bcl2-negative tumors was infrequent, whereas patients with HPV-positive/Bcl2-positive or HPV-negative/Bcl2-negative tumors had intermediate risks compared with the poor prognosis of patients with HPV-negative/Bcl2-positive tumors (Fig. 3).

Patients receiving high-dose or weekly platinum-based therapies did not differ significantly in expression of these biomarkers or in outcome, whereas both HPV and Bcl2 maintained significance in Cox analysis on patients stratified by treatment. These results argue that HPV and Bcl2 are predictors of outcome for advanced OPSCC treated with either type of platinum-based chemotherapy delivered concurrently with radiation. The size and retrospective nature of this study, however, limits our ability to assess clinical equivalence of high-dose versus weekly chemotherapy.

The independence of Bcl2 status from patient characteristics, TNM staging, and HPV status, together with the independent prognostic significance of Bcl2 and HPV, suggests that pretreatment testing of both Bcl2 and HPV (or its surrogate p16) could improve prognostic accuracy in OPSCC treated with this standard of care. The high percentage of HPV-positive tumors that were Bcl2 positive

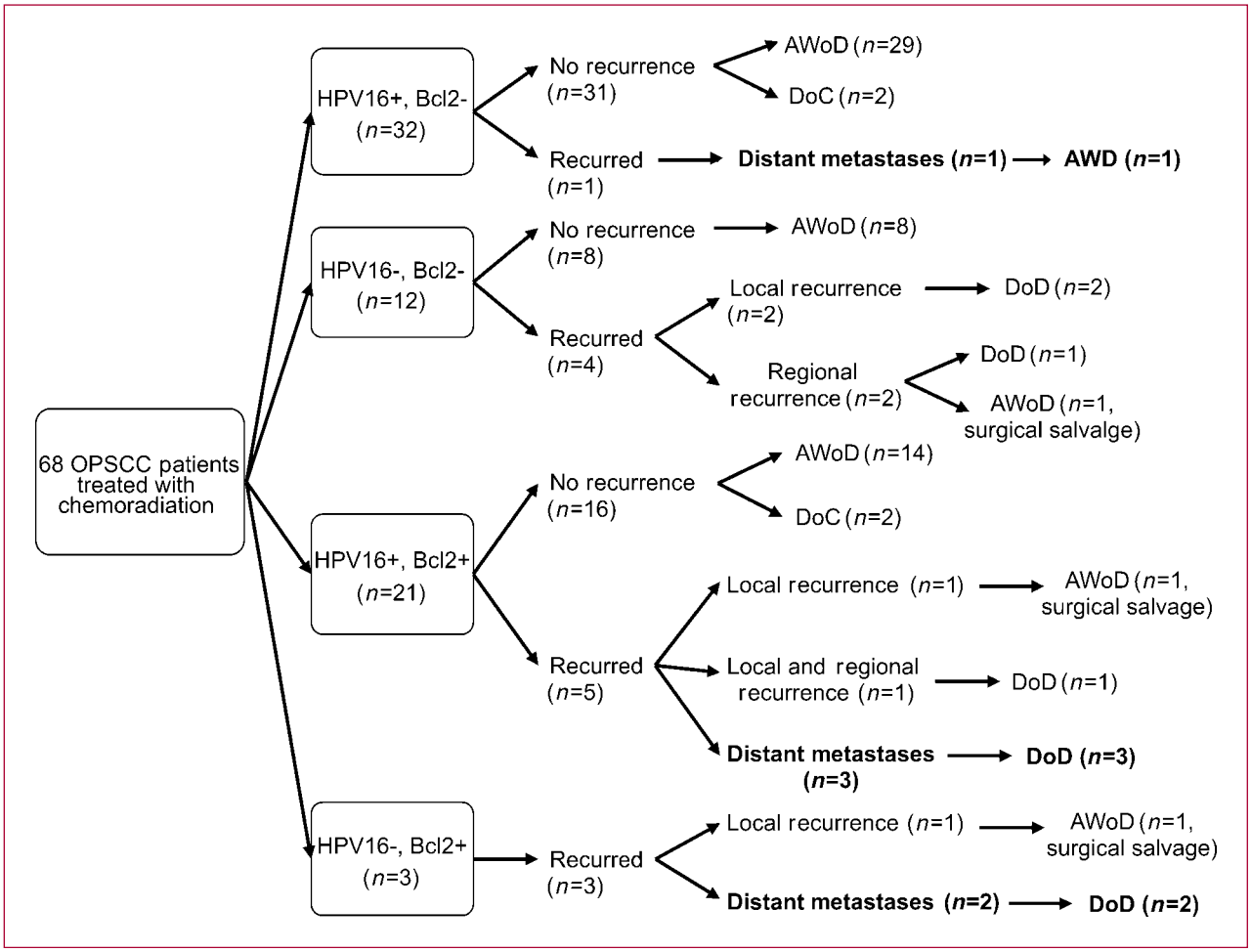

Fig. 2. Diagram of patient outcomes by HPV and Bcl2 status. AWoD, alive without disease; DoC, died of other causes; AWD, alive with disease; DoD, died of disease. Distant metastases are highlighted in bold; HPV16+, HPV16 positive, HPV16–, HPV16 negative.

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(21 of 53, 40%) suggests that Bcl2 will continue to be a useful marker of clinical response as the etiology of OPSCC continues to shift over time from tumors associated with traditional risk factors to HPV-associated tumors, in which traditional TNM staging, smoking, and excessive alcohol use may not reliably predict treatment outcome (3, 11).

Implications for biomarker research in OPSCC. It will be important to extend the present results in a larger, multiinstitutional study. The present study was sufficient to show significant influences of both Bcl2 and HPV on outcome, with important implications for eventually matching treatment regimens to underlying tumor biology in OPSCC. Nevertheless, the size of this study left fairly wide bounds on the magnitudes of the associated hazards, which can be refined in a larger study. A larger study would also allow detailed evaluation of interactions of HPV and Bcl2 status with other biomarkers, including those examined for HNSCC (but not restricted as here to OPSCC treated with concurrent chemoradiation) such as p53 and epidermal growth factor receptor status (6, 8). Additional biomarkers might also identify subsets of Bcl2-positive, HPV-positive tumors that respond differently to chemoradiation, which failed in about one quarter of such patients reported here.

The antiapoptotic Bcl2 family member $BclX_L$ has also been associated with outcome in HNSCC. For example, high $BclX_L$ was associated with poor response to induction chemotherapy in laryngeal cancer (25), but in an OPSCC induction study from the same institution, high $BclX_L$ was only associated with poor outcome in combination with low p53 expression (8). The present study of OPSCC treated without induction did not find a significant association of $BclX_L$ with outcome; we did not, however, examine pretreatment p53 status. There may be differences in the mechanisms that select for high expression of Bcl2 versus $BclX_L$ in different subgroups of HNSCC, or these proteins might provide different protection against particular therapeutic regimens or in particular cellular contexts such as low p53 expression. Because Bcl2 and $BclX_L$ are closely related antiapoptotic proteins and the Bcl2 family inhibitors now being evaluated target both of them (27), both may need to be evaluated until further studies clarify their relative roles.

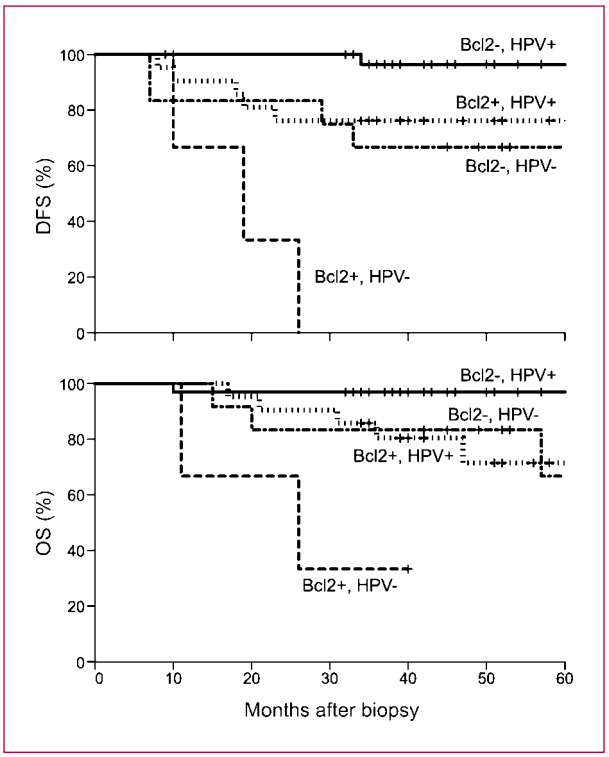

Fig. 3. Kaplan-Meier curves for DFS and OS by HPV and Bcl2 status in pretreatment biopsies. Crosses mark censoring times. Top, DFS; bottom, OS.

Implications for developing treatments for OPSCC. Bcl2 may play a direct role in treatment resistance rather than simply being a marker of outcome. Our initial identification of Bcl2 as a biomarker in OPSCC arose from our studies of the cellular mechanisms underlying apoptosis resistance in HNSCC cell lines (13). High levels of Bcl2 expression provide cisplatin resistance to such cell lines (13, 28) and the well-known antiapoptotic effects of Bcl2 presumably underlie its association with worse outcome in primary radiotherapy of laryngeal cancer (29). The antiapoptotic mechanisms of Bcl2 and related antiapoptotic proteins are well enough understood that Bcl2 family inhibitors are being evaluated in clinical trials (27). Our results suggest that Bcl2 family inhibitors should be evaluated for treating HNSCC, particularly in combination with cytotoxic therapy. Bcl2 family inhibitors might be therapeutically useful regardless of Bcl2 expression levels, similar to the lack of association between epidermal growth factor receptor expression levels and response to cetuximab (30).

In contrast, although HPV-associated HNSCC have better outcome than do tumors associated with traditional risk factors, the biological mechanisms underlying the improved outcome are not well understood (3, 31). The E6 and E7 HPV oncoproteins suppress the actions of the p53 and p16/pRb tumor suppressor pathways, which could reduce selective pressure for loss of these pathways during tumor development. Thus, HPV-associated tumors might be more likely to respond favorably to cytotoxic therapies than do tumors associated with traditional risk factors, in which these tumor suppressor pathways are often lost (32). HPV-associated tumors might also have enhanced immune responses to therapy (15, 31). No therapies directly exploiting the cellular biology of HPV-related tumors are yet available. If they are developed, however, our findings that HPV and Bcl2 status are independent in OPSCC and that these markers have independent prognostic implications following concurrent chemoradiation suggest that such treatments could act synergistically with therapies targeting Bcl2.

The association we found in Bcl2 with distant metastases in the setting of locoregional control suggests that OPSCC patients with Bcl2-positive tumors might benefit

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Table 3. Combining Bcl2 and HPV as predictors of DFS and OS

Analysis	Predictor	DFS		OS	
		HR (95% CI)*	$P^{\dagger}$	HR (95% CI)*	$P^{\dagger}$
Stratified by Bcl2	HPV (+ vs –)	0.13 (0.04-0.42)	<0.001	0.17 (0.04-0.63)	0.009
Stratified by HPV	Bcl2 (+ vs –)	7.6 (1.9-30)	0.004	6.9 (1.7-27)	0.006
Bivariate	HPV (+ vs –)	0.11 (0.035-0.37)	<0.001	0.19 (0.05-0.67)	0.01
	Bcl2 (+ vs –)	6.1 (1.8-21)	0.004	7.4 (1.9-28)	0.004

NOTE: In addition to these direct tests of the hypothesis that HPV and Bcl2 together predict outcome, stepwise forward Cox regression (allowing inclusion of all variables in Table 2) resulted in models with only Bcl2 and HPV as significant ($P < 0.05$) predictors of outcome, both for DFS and for OS.

*CI by Cox proportional hazard analysis.

†Wald test.

from more aggressive systemic chemotherapy. Concurrent regimens rely on combined effects of radiation with chemotherapy locally but offer less systemic chemotherapy to address metastasis than do induction or sequential regimens. This hypothesis could be tested by retrospective sites-of-failure analysis, based on Bcl2 expression and HPV status in pretreatment pathology specimens and outcomes from published phase III trials of induction chemotherapy for head and neck cancer (33, 34). Patients with HPV-negative Bcl2-positive tumors likely require the most aggressive treatment, such as induction followed by concurrent chemoradiation, surgery with postoperative chemoradiation (10), or, when they become available, Bcl2 inhibitors.

Our results support trials to evaluate the role of Bcl2 inhibitors in combination with chemoradiation. It will be important to determine whether Bcl2 or other antiapoptotic Bcl2 family members are the primary targets of such inhibitors in HNSCC. Our results suggest that Bcl2 is specifically associated with resistance to chemoradiation treatment of OPSCC, so Bcl2-specific inhibitors may provide specific targeted therapy with potentially less toxicity than compounds presently undergoing clinical trials (27), which inhibit several antiapoptotic Bcl2 family members.

Our results also have implications for developing less aggressive treatments for HPV-positive OPSCC, such as standard radiation therapy without chemotherapy, concurrent chemotherapy with less-than-standard radiation doses, cetuximab with radiation, or upfront surgery followed by reduced radiation. Further understanding the molecular mechanisms underlying the sensitivity of HPV-positive OPSCC to radiation and chemotherapy should help design less aggressive new treatments. The diminished morbidity expected with less aggressive treatments, however, must be balanced against the currently available almost complete cure rate we found with concurrent chemoradiation of HPV-positive, Bcl2-negative tumors.

In summary, HPV status and Bcl2 expression together define subgroups of OPSCC patients that have differing prognoses after treatment with concurrent chemoradiation. These subgroups should be validated and refined in prospective analysis, and considered for unique approaches to therapy that recognize their low, intermediate, and high risks for relapse after concurrent treatment with radiation and platin-based chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

The Laurence Murphy Fund, the Norman Knight Fund, the James Kelly Fund for Head and Neck Research, and the Flight Attendant Medical Research Institute (J.W. Rocco).

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Received 12/07/2009; revised 01/25/2010; accepted 01/26/2010; published OnlineFirst 03/16/2010.

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Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

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Published OnlineFirst March 16, 2010; DOI: 10.1158/1078-0432.CCR-09-3185

Clinical Cancer Research

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Clin Cancer Res 2010;16:2138-2146. Published OnlineFirst March 16, 2010.