

Cyclin D1 Is a Valuable Prognostic Marker in Oropharyngeal Squamous Cell Carcinoma

Ziwei Yu,¹ Paul M. Weinberger,¹ Bruce G. Haffty,² Clarence Sasaki,¹ Cynthia Zerillo,³ John Joe,¹ Diane Kowalski,⁴ James Dziura,⁵ Robert L. Camp,⁴ David L. Rimm,⁴ and Amanda Psyrrri³

Departments of ¹Otolaryngology, ²Therapeutic Radiology, ³Medical Oncology, ⁴Pathology and ⁵Biostatistics, Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

Background: The current tumor-node-metastasis system is inadequate to accurately classify patients in terms of prognosis. Thus, with the availability of recently developed molecular tools, considerable interest lies in discovering prognostic markers in order to guide treatment decisions. In this study, we sought to determine the prognostic significance of the cell cycle regulator cyclin D1 in oropharyngeal squamous cell carcinoma (OSCC).

Experimental Design: We studied the protein expression levels of cyclin D1 on a tissue microarray composed of 63 OSCCs with long-term follow-up data available. Protein expression was analyzed with an automated *in situ* quantitative (AQUA) method which allows preservation of tissue morphology while quantifying protein expression in paraffin-embedded tissue.

Results: The mean follow-up time was 35 months. High cyclin D1 nuclear expression was associated with increased 5-year local recurrence rate (48% versus 15%), inferior 5-year disease-free survival (16% versus 58%), and inferior 5-year overall survival (17% versus 53%). In multivariate Cox regression, high nuclear cyclin D1 expression was an independent predictor for local recurrence, disease-free survival, and overall survival at 5 years.

Conclusions: Our results indicate that quantitative assessment of nuclear cyclin D1 expression level by automated *in situ* quantitative analysis is a strong predictor for outcome in OSCC. Thus, cyclin D1 may be a potential target for molecular intervention in patients with oropharyngeal squamous cell cancer.

INTRODUCTION

An estimated 28,260 new cases of oropharyngeal squamous cell carcinoma (OSCC) are expected in the United States in 2004, and about 7,230 of these patients will succumb to the disease (1). Despite numerous advances in diagnosis and treatment over the last several decades, mortality rates have essentially remained unchanged. Even cured patients suffer significant speech and swallowing impairment due to treatment. Oropharyngeal squamous cell cancers represent a heterogeneous group of tumors in terms of the histology, biology, and clinical behavior. Therefore, considerable interest lies in discovering prognostic markers in order to guide treatment decisions. The advances in molecular biology with the development of genomic and proteomic approaches have revolutionized our ability to study the molecular signatures of tumors.

Recently, genetic alterations and the expression of cell cycle regulatory gene products have been studied in various malignancies. Cell cycle regulators (p53, p16, cyclin D1, CDK2, and p34^{cdc2}; refs. 2–6), key components of pathways that control tumor behavior (epidermal growth factor receptor; refs. 7, 8), angiogenesis markers (vascular endothelial growth factor; ref. 9), adhesion molecules (CD44; ref. 10) and markers of invasion and metastasis (matrix metalloproteinases; refs. 11, 12) have been examined for prognostic significance in head and neck squamous cell cancers (HNSCC). Results for each of these markers are mixed; this reflects the complexity of tumor biology and the multitude of factors that contribute to the diversity of clinical presentation of disease.

Molecular studies in HNSCC have indicated that 11q13 amplification is a frequent event. The protooncogene *cyclin D1* resides at this area and is found to be amplified in about 30% of HNSCC (13). Cyclin D1 is a cell cycle regulatory protein that binds cyclin-dependent kinase 4 (Cdk4) and promotes phosphorylation of the retinoblastoma protein (14). Retinoblastoma protein phosphorylation is required for progression through the G₁-S cell cycle checkpoint. Several studies in HNSCC (3, 15–18), especially in laryngeal cancers, have shown the adverse impact of cyclin D1 gene amplification and/or protein overexpression on patient outcome.

Here, we sought to determine the prognostic significance of cyclin D1 in OSCC using an automated *in situ* quantitative (AQUA) method of protein expression analysis on a tissue array composed of 96 OSCC with long-term patient follow-up data. Our study shows an independent prognostic significance of cyclin D1 protein levels in determining patient outcome.

MATERIALS AND METHODS

Tissue Microarray Construction. The cohort was assembled from patients with primary oropharyngeal cancer of squamous cell histology treated at Yale-New Haven hospital between 1980 and 1999. Patients were treated with primary external beam radiotherapy (EBRT) or gross total surgical resection and postoperative radiotherapy. Exclusion criteria

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Requests for reprints: Amanda Psyrrri, Yale Cancer Center, P.O. Box 208032, New Haven, CT 06520. Phone: 203-737-2476; Fax: 203-785-7531; E-mail: diamando.psyrrri@yale.edu.

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included presentation with recurrent or metastatic disease, or failure to receive a full course of radiation therapy. Following Institutional Review Board approval, the tissue microarray was constructed as previously described (19), including 96 cases that met inclusion criteria. Paraffin-embedded formalin-fixed tissue blocks from the Yale University Department of Pathology archives were obtained and H&E counterstained. These slides were reviewed by a pathologist (D. Kowalski) to select representative areas of invasive tumor to be cored. The cores were taken using 0.6 mm² blunt-tip needles and placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have shown that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochemistry (20–22). The tissue microarray was then cut to yield 5- μ m sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Quantitative Immunohistochemistry. Tissue microarray slides were deparaffinized and stained as previously described. In brief, slides were deparaffinized with xylene followed by ethanol. Following rehydration in distilled water, antigen retrieval was accomplished by pressure-cooking in 0.1 mol/L citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating in 0.3% hydrogen peroxide in methanol for 30 minutes. Nonspecific antibody binding was then blocked with 0.3% bovine serum albumin for 30 minutes at room temperature. Following these steps, slides were incubated with primary mouse monoclonal antibody to cyclin D1 at 1:250 dilution (ab6152, Abcam, Cambridge, MA) at 4°C overnight. Subsequently, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision, DAKO Corp.) for 1 hour at room temperature. Tumor cells were identified by use of anticytokeratin antibody cocktail (rabbit anti-pancytokeratin antibody z0622, DAKO) with subsequent goat anti-rabbit antibody conjugated to Alexa546 fluorophore (A11035, Molecular Probes, Eugene, OR). We added 4',6-diamidino-2-phenylindole to visualize nuclei. Target (cyclin D1) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide, Perkin-Elmer, Corp.). Cy-5 (red) was used because its emission peak is well outside the green-orange spectrum of tissue autofluorescence. Slides were mounted with a polyvinyl alcohol-containing aqueous mounting media with antifade reagent (*n*-propyl gallate, Acros Organics, Fairlawn, NJ).

Automated Image Acquisition and Analysis. Automated image acquisition and analysis using AQUA has been described previously (23). In brief, monochromatic, high-resolution (1,024 × 1,024 pixel; 0.5 μ m) images were obtained of each histospot using filter cubes specific to the emission/excitation spectra of 4',6-diamidino-2-phenylindole (358/461 nm), Alexa 546 (556/573 nm), and Cy-5 (650/670 nm) (Optical Analysis, New Hampshire). We distinguished areas of tumor from stromal elements by creating a mask from the cytokeratin signal (in this case identified by Alexa 546 signal). A tumor nuclei-specific compartment was created by using 4',6-diamidino-2-phenylindole signal to identify nuclei within the previously defined tumor mask. Overlapping pixels (to a 99% confidence interval)

were excluded from the nuclear compartment. The cyclin D1 signal (AQUA score) was scored on a normalized scale of 1 to 255 expressed as pixel intensity divided by the target area (tumor nuclei compartment). AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor (we have previously shown that the tissue microarray technique, with 2-fold redundancy, is a valuable and accurate method for analysis of protein expression in large archival cohorts; ref. 21).

Western Blotting. The cyclin D1 antibody used for immunohistochemistry was validated using Western blot analysis. Nuclear cell lysate from C32 cell lines (Santa Cruz Biotechnology, CA) and BT474 total cell lysate were used as positive controls for cyclin D1 expression. Protein extracts were resolved by electrophoresis in 10% SDS gels, transferred to nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in 12.5 mmol Tris-0.1 mol/L glycine-20% methanol transfer buffer, and blocked in 5% milk-TBST buffer [5% nonfat dry milk, 25 mmol Tris-HCl (pH 8.0), 125 mmol NaCl, and 0.1% Tween] for 1 hour at 4°C. The membranes were then incubated with mouse monoclonal cyclin D1 antibody (ab6152, Abcam) at 1:500 dilution in TBST overnight at 4°C. The membranes were then washed in TBST five times for 5 minutes at room temperature and then incubated in a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences, Piscataway, NJ) in TBST buffer for 1 hour at room temperature. The membranes were then washed again in TBST five times for 5 minutes at room temperature and incubated with Enhanced Chemiluminescence+ (Amersham Biosciences) and the signals were detected by Hyperfilm (Amersham Biosciences).

Statistical Analysis. Histospots containing <10% tumor, as assessed by mask area (automated), were excluded from further analysis. Previous studies have shown that the staining from a single histospot provides a sufficiently representative sample for analysis. Addition of a duplicate histospot, although not necessary, provides improved reliability. Disease-free survival, overall survival and local recurrence were assessed by Kaplan-Meier analysis with log-rank for determining statistical significance. For survival analysis, automated AQUA scores were converted to binomial variables of high versus low expression around the median. Local recurrence was defined as time from day of diagnosis to development of locally recurrent disease. Overall survival was defined as time from day of diagnosis to death from any cause. Disease-free survival was defined as time from day of diagnosis to the first of either death from any cause or disease progression (local and/or regional recurrence or distant metastases). Univariate survival analysis was also done with the Cox proportional hazards model using cyclin D1 expression as a continuous variable. The multivariate Cox proportional hazards model was used to determine the independent prognostic value of cyclin D1 expression status, management (EBRT alone versus postoperative EBRT), tumor-node-metastasis (TNM) stage, subsite of tumor (within oropharynx), and histologic grade. A multivariate model using cyclin D1 as a continuous variable was also utilized. All survival analyses were done at 5-year cutoffs. Comparison of cyclin D1 expression status with the clinical and pathologic variables gender, TNM stage, histologic grade, management (primary EBRT versus primary surgical excision

plus radiotherapy), oropharyngeal subsite was made using χ^2 analysis. All calculations and analyses were done with Statistical Package for the Social Sciences 11.5 for windows (SPSS, Inc., Chicago, IL) and where appropriate were two-tailed.

RESULTS

Clinical and Pathologic Variable Analysis. There were 96 patients of primary oropharyngeal carcinoma in this cohort that met inclusion criteria, 75 were male and 21 female, with ages ranging from 41 to 79 years old. Eleven patients (11.5%) were at TNM stage II, 27 (28.1%) were at stage III, and 58 (60.4%) were at stage IV. Oropharyngeal subsites included 41 (42.7%) tonsillar fossae, 50 (52.1%) base of tongue, 3 (5.2%) other oropharynx, and 2 (2.1%) not recorded. Fifty-eight (60.4%) patients were managed with primary EBRT, 36 (37.5%) with surgical excision followed by postoperative EBRT, and 2 (2.1%) were not recorded; 21 patients (21.9%) were treated with chemotherapy. For histologic grade, 6 (6.3%) tumors were well differentiated, 44 (45.8%) were moderately differentiated, 35 (36.5%) were poorly differentiated, and 11 (11.5%) not recorded. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Quantitative Immunohistochemistry for Cyclin D1 Protein Expression. Of the 96 patients included in this study, 63 (66%) had sufficient tissue for analysis of cyclin D1 protein expression by AQUA. As visualized by fluorescent immunohistochemistry, cyclin D1 displayed predominately nuclear staining (Fig. 1). Normalized AQUA scores for nuclear cyclin D1 expression were represented on a 1:255 scale. There was an excellent correlation between the two AQUA scores obtained of different tissue sections obtained from the same tumor (Pearson $R = 0.853$). There was no association between cyclin D1 cytoplasmic expression and any of the clinicopathologic variables.

Survival Analysis. Univariate Analysis. Nuclear expression of cyclin D1 as determined by AQUA was examined for association with 5-year local recurrence, disease-free survival and overall survival. For Kaplan-Meier analysis, nuclear AQUA

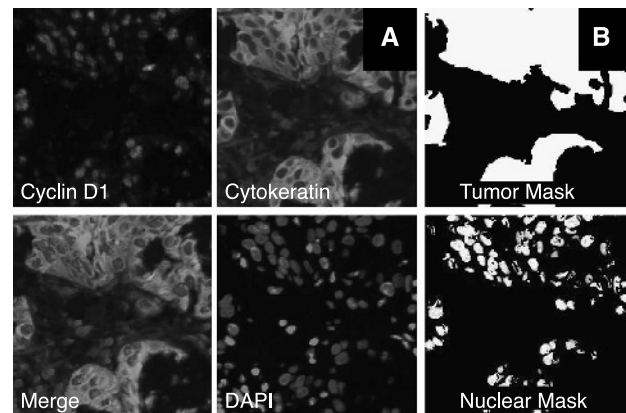


Fig. 1 Immunofluorescence and AQUA analysis of cyclin D1. *A*, the majority of tumors displayed primarily nuclear localization of cyclin D1 as visualized by fluorescent immunohistochemistry. Cyclin D1 is shown in red, cytokeratin in green, and nuclei (as visualized by 4',6-diamidino-2-phenylindole) in blue. A merged image is also shown. *B*, the AQUA method allows quantitative *in situ* protein expression analysis, including subcellular localization. It also removes a possible confounding factor common to immunohistochemical analysis—surrounding desmoplastic stroma. This is eliminated by using a tumor mask (based on the cytokeratin image) to exclude stromal elements from analysis. A tumor-specific nuclear compartment is generated from the 4',6-diamidino-2-phenylindole signal within the tumor mask. Target expression is then calculated within the compartmental masks.

scores were split around the median (14.3 AQUA units) to yield two groups, low versus high expressors. Kaplan-Meier survival curves for overall survival, disease-free survival, and local recurrence are given in Fig. 2. Patients with high nuclear cyclin D1 expression showed inferior disease-free survival (16.67% versus 67.74; $P = 0.0019$), overall survival (19.35% versus 67.74; $P = 0.0134$) and increased risk of local recurrence (43.33% versus 12.9%; $P = 0.0128$). To assess the prognostic value of nuclear cyclin D1 expression on a continuous scale, univariate survival analysis was also done using the Cox proportional hazards model. Nuclear cyclin D1 as a continuous variable was a significant predictor for local recurrence ($P = 0.008$), disease-specific survival ($P = 0.043$), and overall survival ($P = 0.014$). Results of Cox univariate analysis are summarized in Table 2.

Multivariate Analysis. Using the Cox proportional hazards model, multivariate analyses were done to assess the independent predictive value of cyclin D1 expression groups (high versus low) for local recurrence, disease-free survival, and overall survival. The following prognostic variables were also included: subsite within oropharynx, TNM stage, management (EBRT versus primary surgical excision plus radiotherapy), and histologic grade. For overall survival and disease-free survival, only nuclear cyclin D1 expression remained an independent prognostic factor ($P = 0.007$ and $P = 0.001$, respectively). For local recurrence, both nuclear cyclin D1 expression ($P = 0.012$) and management ($P = 0.014$) were independent prognostic factors. Cox multivariate regression was also done using cyclin D1 as a continuous variable. In this model, cyclin D1 as a continuous variable remained an independent predictor of overall survival ($P = 0.003$), disease-free survival ($P = 0.026$), and local

Table 1 Demographic, clinical, and pathologic data

Age	41-79 (median 61.5)	
Gender	Male	75
	Female	21
Site	Tonsillar fossae	41
	Base of tongue	50
	Other oropharynx	3
TNM stage	II	11
	III	27
	IV	58
	Grade	Well differentiated
	Moderately differentiated	44
	Poorly differentiated	35
Management	Primary radiotherapy	58
	Post-op radiotherapy	36
Chemotherapy	Yes	21
	No	75

NOTE. Clinicopathologic data was not available for some patients: site (2), grade (11), management (2).

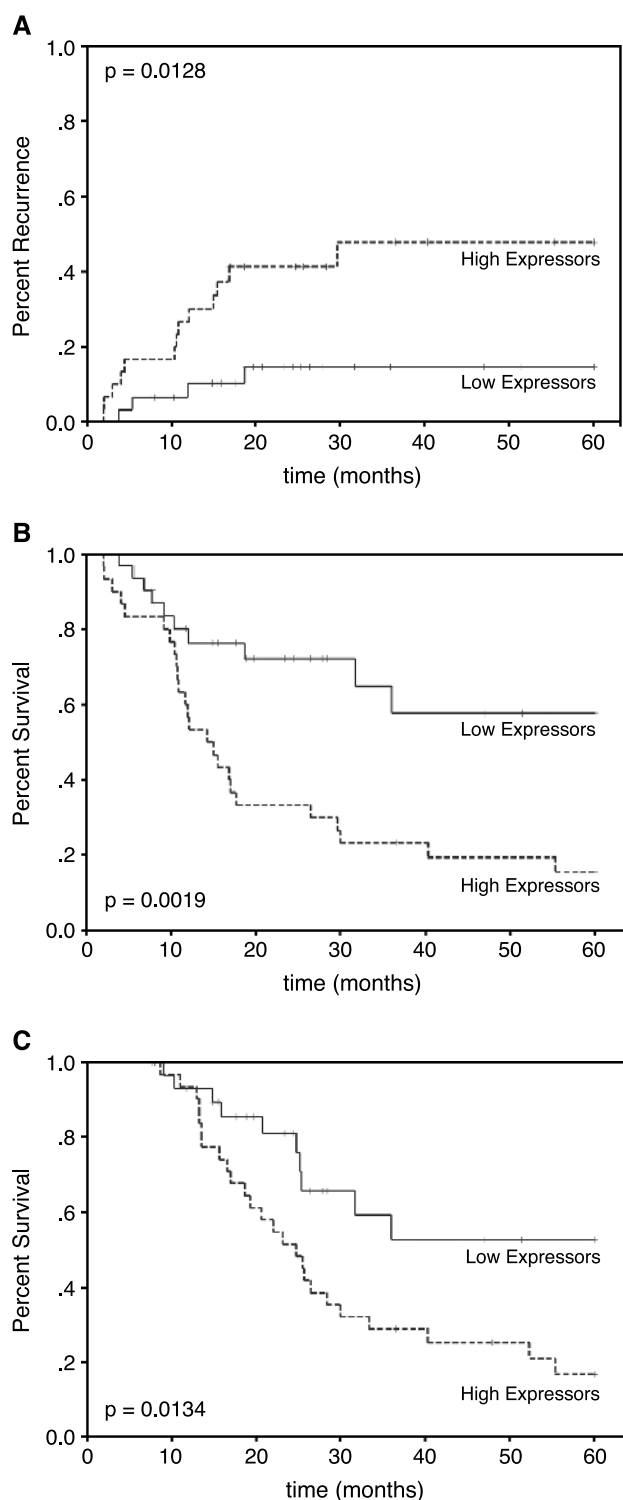


Fig. 2 Kaplan-Meier estimates of 5-year local recurrence, disease-free survival, and overall survival by cyclin D1 nuclear expression. Patients with high nuclear cyclin D1 expression showed increased local recurrence (A), inferior disease-free survival (B), and overall survival (C) compared with patients with low nuclear cyclin D1 expression.

Table 2 Univariate survival analysis by Cox proportional hazards model

Variable	<i>B</i>	SE	<i>P</i>
Local recurrence*			
Cyclin D1 nuclear AQUA score	0.010	0.004	0.008†
Disease-free survival*			
Cyclin D1 nuclear AQUA score	0.007	0.003	0.043‡
Overall survival			
Cyclin D1 nuclear AQUA score	0.010	0.004	0.014‡

NOTE. Univariate Cox analysis was done using AQUA score as a continuous variable. As such, a hazard ratio between two hypothetical patients can be estimated from *B* using the equation: hazard ratio = $e^{(B \times \Delta\text{AQUA})}$ where ΔAQUA is the difference in AQUA score between the two patients. For example, given two patients with nuclear cyclin D1 AQUA scores of 50 and 100, the hazard ratio for local recurrence between them would be approximately $e^{(0.01 \times 50)}$, or 1.65 (a 165% increased risk of local recurrence for a 50-point difference).

*One patient lacked recurrence data and was excluded from local recurrence and disease-free survival calculations.

†Significant at the 0.01 level.

‡Significant at the 0.05 level.

recurrence ($P = 0.031$). Results of the multivariate analyses are indicated in Table 3.

Western Blotting. As shown in Fig. 3, incubation of C32 nuclear and BT474 total cell extracts with antibody to cyclin D1 (ab6152 MonoClonal Ab, Abcam) recognized a band of 32 kDa, consistent with cyclin D1 protein.

DISCUSSION

Our study shows that cyclin D1 protein expression levels as judged by quantitative immunohistochemistry are inversely correlated with outcome in oropharyngeal squamous cell cancers. In multivariate analysis, adjusted for well-recognized prognostic indicators, cyclin D1 maintained its independent prognostic value.

Cyclin D1 amplification is one of the most frequent molecular alterations in HNSCC (13). Cyclin D1 gene amplification has been shown to correlate with protein overexpression as assessed by immunohistochemistry (15). However, protein overexpression may also occur via unknown mechanisms which precede gene amplification such as translocations, inversions, or yet unknown causes of transcriptional activation (15, 24, 25). As a result, in HNSCC, the incidence of protein overexpression reported is higher than the rate of gene amplification [39-59% (refs. 26, 27) and 26%-39% (refs. 26, 28), respectively]. Some studies also report a difference in cyclin D1 expression among different sites within the head and neck, with hypopharynx demonstrating the highest levels (26, 27).

Several studies have examined the prognostic significance of cyclin D1 overexpression in HNSCC. Dong et al. (29), found that coexpression of cyclin D1 and CDK4 by immunohistochemistry was associated with the poorest overall survival in a cohort of 102 patients with laryngeal carcinoma. Pignataro et al. (30), found that cyclin D1 overexpression by immunohistochemistry was an independent predictor of adverse disease-free survival in a cohort of 149 patients with laryngeal carcinomas treated with surgery and radiotherapy. Bova et al. (16), also

Table 3 Multivariate 5-year survival analysis by Cox regression

Variable	Hazard ratio	95% Confidence interval	P
Local recurrence*			
Management	0.156	0.04-0.69	0.014†
Histologic grade	0.440	0.15-1.30	0.14
TNM stage	1.551	0.63-3.84	0.34
Subsite within oropharynx	0.837	0.47-1.48	0.54
Low nuclear cyclin D1 expression	6.011	1.47-24.55	0.012‡
Disease-free survival*			
Management	0.866	0.36-2.07	0.75
Histologic grade	0.758	0.36-1.58	0.46
TNM stage	1.360	0.73-2.55	0.34
Subsite within oropharynx	0.752	0.51-1.11	0.15
Low nuclear cyclin D1 expression	3.897	1.71-8.90	0.001‡
Overall survival			
Management	0.867	0.36-2.10	0.75
Histologic grade	0.632	0.32-1.26	0.19
TNM stage	1.566	0.86-2.85	0.14
Subsite within oropharynx	0.781	0.53-1.16	0.22
Low nuclear cyclin D1 expression	3.325	1.40-7.92	0.007‡

*One patient lacked recurrence data and was excluded from local recurrence and disease-free survival calculations.

†Significant at the 0.05 level.

‡Significant at the 0.01 level.

reported that cyclin D1 overexpression is an independent predictor for disease-specific death in a cohort of 148 patients with carcinoma of the anterior tongue. In a similar fashion, Kyomoto et al. (17), reported that cyclin D1 amplification by differential PCR method and protein overexpression by immunohistochemistry was associated with poor outcome in 45 paraffin-embedded sections from HNSCC. The authors also found that gene amplification retained an independent prognostic value, whereas protein overexpression did not. Namazie et al. (31), using fluorescent *in situ* hybridization, reported that cyclin D1 amplification and p16 deletion together correlated with recurrence, distant metastasis, and survival in a cohort of 103 HNSCC. Contrary to the aforementioned reports, Fortin et al. (32), studied prospectively chromosome 11q13 gene amplifications in 50 oral and oropharyngeal carcinomas for their predictive value in subclinical lymph node metastases or disease recurrence. The authors found no association between 11q13 gene amplification and subclinical lymph node invasion. We also

studied the present cohort for cyclin D1 overexpression by conventional immunohistochemistry and reported no prognostic value (33).

The present study is the only one of its kind that uses a quantitative method of protein analysis to evaluate the prognostic significance of cyclin D1 in oropharyngeal cancer. Because cyclin D1 protein overexpression may occur via other mechanisms besides gene amplification, we believe that measurement of protein levels will be more informative than cyclin D1 DNA copies. However, measurement of protein levels with conventional immunohistochemistry often fails to provide accurate results. The discrepancy between the results we obtained with AQUA and conventional immunohistochemistry shows one of the problems with by-eye scoring of immunohistochemical stains; i.e., the difficulty humans have in translating a continuous marker into a nominal four-point scale. Specifically, the pathologist tends to group things as positive or negative, whereas the automated device results in continuous full-scale scores.

Our finding of lower percentage of stage IV tumors (55% versus 65% for stage III) expressing high levels of cyclin D1 deserves mention. Besides tobacco and alcohol use, high-risk human papillomaviruses account for squamous cell carcinoma development in the oropharyngeal location (34). The E6 and E7 genes of the high-risk human papillomaviruses encode oncoproteins that target the p53 and retinoblastoma tumor suppressors, respectively, for degradation (35, 36). Cyclin D1 expression is positively regulated by retinoblastoma protein (37, 38) and retinoblastoma protein degradation by E7 results in down-regulation of cyclin D1 (39, 40). Human papillomavirus-associated oropharyngeal tumors usually present in advanced stage (41). Thus, our lower incidence of cyclin D1 expression in stage IV tumors may be explained by human papillomavirus involvement in the pathogenesis of oropharyngeal cancers.

HNSCC represent a heterogeneous group of tumors in terms of biology, etiology, and clinical behavior. Molecular alterations occur with different frequencies in different head and neck sites (42–44). For example, Begum et al. (42), studied

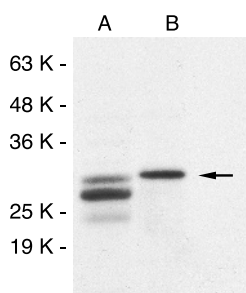


Fig. 3 Western blot validation of antibody. Nuclear cell lysate from C32 cell lines (A) and BT474 total cell lysate (B) were used as positive controls for cyclin D1 expression. Membranes were probed with cyclin D1 antibody (ab6152, Abcam) at 1:500 dilution overnight at 4°C. The cyclin D1 antibody successfully recognized a band at 32 kDa, consistent with cyclin D1 protein. The two bands represent the two forms of cyclin D1 in the cell: the hypophosphorylated and phosphorylated cyclin D1 fractions. The antibody to cyclin D1 recognizes both forms.

68 HNSCC and found that 24 of 31 oropharyngeal tumors were p16-positive by immunohistochemistry, whereas only 1 of 37 non-oropharyngeal tumors was p16-positive. Cyclin D1 overexpression has also been found to be site-specific (44). Therefore, the relative contribution of cyclin D1 overexpression in oropharyngeal carcinogenesis, in relation to the other markers previously described, cannot be determined until the latter are examined in large cohorts of OSCC patients.

Using AQUA we found that cyclin D1 overexpression is a powerful independent predictor for adverse outcome in OSCCs. This result shows the power of AQUA over conventional immunohistochemistry for discovering molecular prognostic indicators. A comparison of the incidence of cyclin D1 overexpression by AQUA with that of gene amplification by real-time PCR is being undertaken in our laboratory.

In addition to identifying a strong independent predictor for outcome in OSCC, our findings also have important therapeutic implications: strategies to counteract the effect of cyclin D1 overexpression such as cyclin-dependent kinase inhibitors may hold promise for treatment of patients with OSCC.

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