

β -Catenin Functions Mainly as an Adhesion Molecule in Patients with Squamous Cell Cancer of the Head and Neck

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Abstract **Background:** β -catenin, depending on subcellular localization, plays a dual role in carcinogenesis: as a signaling factor (in the nucleus) and as an adhesion molecule (in cell membrane). In this study, we sought to determine the role of β -catenin in head and neck carcinogenesis. **Methods:** First, we studied the incidence of mutations of β -catenin in a cohort of 60 head and neck squamous cell cancers (HNSCC). We subsequently evaluated the protein expression levels of β -catenin in a cohort of oropharyngeal squamous cell cancer tissue microarray using a novel *in situ* method of quantitative protein analysis and correlated those with cyclin D1 levels and clinical and pathologic data. **Results:** The mean follow-up time for survivors was 45 months and for all patients was 35 months. We found no mutations in the cohort of 60 HNSCC. β -catenin displayed primarily membranous expression pattern. Patients with high tumor-node-metastasis stage were more likely to have high expression of β -catenin ($P = 0.040$). Patients with low β -catenin expression had a local recurrence rate of 79% compared with 29% for patients with high β -catenin tumors ($P = 0.0021$). Univariate Cox regression revealed a hazard ratio for low β -catenin tumors of 3.6 ($P = 0.004$). Kaplan-Meier analysis showed that patients with low β -catenin expressing tumors trended toward worse 5-year disease-free survival ($P = 0.06$). In multivariate analysis, only β -catenin expression status was an independent prognostic factor ($P = 0.044$) for local recurrence. Tumors with high β -catenin had low cyclin D1 and vice versa ($P = 0.007$). **Conclusions:** The absence of activating β -catenin mutations combined with the inverse correlation between β -catenin levels with cyclin D1 levels and outcome suggest that β -catenin mainly functions as an adhesion and not signaling molecule in HNSCC.

Despite advances in diagnosis and treatment, morbidity and mortality rates of patients with head and neck squamous cell cancer (HNSCC) have essentially remained unchanged over the past several decades (1). In addition, the inability of the current tumor-node-metastasis (TNM) stage to accurately classify patients in relation to prognosis has prompted a search for molecular markers that accurately predict outcome. The advances in molecular biology with the availability of genomic and proteomic approaches have dramatically enhanced our ability to elucidate key components of pathways that control tumor behavior.

Alterations in cell fate, motility, and adhesion through dysregulation of signaling pathways are hallmarks of cancer in which cells are unresponsive to normal regulatory cues from their microenvironment. Of the many regulatory factors involved in these events, β -catenin is particularly interesting because it functions both as a component of cadherin-catenin adhesion system and as signaling molecule (2). β -catenin binds to the cytoplasmic domain of type I cadherins and regulates cell-to-cell adhesion (3–5). β -catenin can also form a complex with axin and adenomatous polyposis coli protein and undergoes degradation in the 26S proteasome (6, 7). Wnt signaling inhibits β -catenin proteasomal degradation leading to β -catenin stabilization and accumulation in the cytoplasm (7). β -catenin mutations, which result in β -catenin stabilization and cytoplasmic accumulation, have also been described (8). The accumulation of cytoplasmic (signaling) β -catenin leads to its nuclear localization where it binds to T-cell factor/lymphoid enhancer factor family of transcription factors and induces expression of target genes, such as *cyclin D1* (7). Thus, depending on subcellular localization, β -catenin plays a dual role in carcinogenesis: as a signaling factor (in the nucleus) and as an adhesion molecule (in cell membrane).

As β -catenin plays an important role in carcinogenesis, we sought to determine the role of β -catenin in HNSCC. First, we studied the incidence of mutations of β -catenin in a cohort of 60 HNSCCs. Finding none, we subsequently evaluated the

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protein expression levels of β -catenin in a cohort oropharyngeal squamous cell cancer tissue microarray using a novel *in situ* method of quantitative protein analysis and correlated those with cyclin D1 levels and clinical and pathologic data.

Materials and Methods

Patient selection. Following institutional review board approval, the following cohorts of patients were assembled. The cohort for mutational analysis was assembled from the Yale University Cytology Tissue Bank collection of fixed fine needle aspiration cytology specimens. Specimens were reviewed by a pathologist for diagnosis and location of lesion. All were from palpable lesions from the head and neck assigned the diagnosis of squamous cell carcinoma. The cohort for automated image acquisition and analysis (AQUA) was subsequently 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 included presentation with recurrent or metastatic disease or failure to receive a full course of radiation therapy.

Mutation analysis. DNA was harvested from cytology specimens using standard methods. Briefly, cell pellets from 1 to 2 mL cytology specimens (~50-100 μ L cell material) were resuspended in 200 μ L digestion buffer (10 mmol/L Tris 8.0, 100 mmol/L NaCl, 25 mmol/L EDTA, 0.5% Tris, and 100 μ g/ μ L proteinase K) at 37°C overnight. Following digestion, DNA was purified with phenol/chloroform extraction followed by ethanol precipitation. DNA was resuspended in Tris-EDTA buffer (pH 7.4). The 200 bp amplicon of β -catenin exon 3 and the 300 bp amplicon of plakoglobin exon 3 was amplified by standard PCR using AmpliTaq gold polymerase enzyme (Perkin-Elmer, Wellesley, MA). These locations were chosen based on the key regulatory serine/threonine residues located within them. Primers were β -catenin forward 5'-GCTGATTTGATGGAGTTGGA-3' and β -catenin reverse 5'-GCTACTTGTCTTGAGTGAA-3'. Primers for plakoglobin were forward 5'-GAGACCCCTACAATCTGCCTCCTTCA-3' and plakoglobin reverse 5'-GGAGCAGCCTATCAAGGTGACTGACTGG-3'. Samples with adequate PCR product were analyzed by single-stranded conformational polymorphism mutation analysis. Briefly, 40 ng PCR samples were diluted in 2.5 to 5 volumes single-stranded conformational polymorphism stop solution (95% formamide, 5.0 mol/L NaOH, 0.1% bromophenol blue, and 0.1% xylene cyanol), boiled, and snap cooled on ice. Samples were loaded on a 40% Mutation Detection Enhancement vertical gel (FMC BioProducts, Rockland, ME) run at 400 V, 4 to 6 hours at 18°C. Gels were soaked in Syber green II at 1:10,000 in Tris-EDTA for 10 to 200 minutes (FMC BioProducts) and visualized on an UV lightbox. Candidate bands were excised and hydrated in 50 μ L Tris-EDTA. DNA collected from the gel underwent PCR for β -catenin exon 3 or plakoglobin as above. Recovered PCR product was cleaned by spin column purification (QIAquick, Qiagen, Valencia, CA) and sent for sequencing (Keck Facility, Yale University).

Tissue microarray construction. For protein expression studies using AQUA, paraffin-embedded specimens are required; cytology specimens are inadequate. Therefore, a tissue microarray was constructed as previously described, including 94 cases that met inclusion criteria for AQUA analysis. Tissue cores were obtained from paraffin-embedded formalin-fixed tissue blocks from the Yale University Department of Pathology archives. Slides from all blocks were reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Cores from Caski cell lines fixed in formalin and embedded in paraffin were selected for positive controls and included in the array. Additionally, 53

histologically confirmed normal squamous epithelium samples from formalin-fixed and paraffin-embedded skin were included for comparison of β -catenin expression in normal tissue. The tissue microarray was then cut to 5 μ m sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

Immunofluorescence. 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 in TBS (pH 8.0) for 30 minutes at room temperature. Following these steps, slides were incubated with mouse monoclonal primary antibody at 4°C overnight. Primary antibody to β -catenin (clone14, BD Transduction Laboratories, San Jose, CA) was used at 1:450 dilution in 0.3% bovine serum albumin/TBS. This antibody has been extensively validated in previous studies using immunohistochemistry and Western blot analysis of normal and neoplastic tissue (9, 10). Primary antibody to cyclin D1 (ab6152, Abcam, Cambridge, United Kingdom) was used at 1:250 dilution in 0.3% bovine serum albumin/TBS. This antibody has been validated by Western blot and immunofluorescence (11). Subsequent to primary antibody incubations, slides were incubated with goat anti-mouse secondary antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision, DAKO Corp., Carpinteria, CA) 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. Antibody labeled target (β -catenin or cyclin D1) molecules were visualized with a fluorescent chromogen (Cy-5-tyramide, Perkin-Elmer). 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, Geel, Belgium).

Automated image acquisition and analysis. Automated image acquisition and analysis using AQUA has been described previously (12). In brief, monochromatic, high-resolution (1,024 \times 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, Nashua, NH). 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 β -catenin and cyclin D1 signal (AQUA scores) were reported on a normalized scale of 1 to 255 expressed as pixel intensity divided by the target area (tumor mask for β -catenin and tumor nuclei mask for cyclin D1). AQUA scores for duplicate tissue cores were averaged to obtain a mean AQUA score for each tumor.

Statistical analysis. Histospots containing <10% tumor 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. AQUA scores represent expression of a target protein on a continuous scale from 1 to 255. It is often useful to categorize continuous variable to stratify patients into high versus low categories. Several methods exist to determine a cutpoint, including biological determination, splitting at the median,

and determination of the cutpoint that maximizes effect difference between groups. If the latter method (the so-called "optimal *P* value" approach) is used, a dramatic inflation of type I error rates can result (13). A recently developed program, X-Tile, allows determination of an optimal cutpoint while correcting for the use of minimum *P* statistics (14). As the AQUA technology is new, there are no established cutpoints available for quantitative β-catenin expression. Therefore, for categorization of β-catenin expression levels, the X-Tile program was used to generate an optimal cutpoint. Two methods of statistical correction for the use of minimal *P* approach were utilized. First, the X-Tile program output includes calculation of a Monte Carlo *P* for the optimal cutpoint generated. Cutpoints that yield Monte Carlo *P* < 0.05 are considered robust and unlikely to represent type I error. Second, the Miller-Siegmund minimal *P* correction referenced by Altman et al. (13) was utilized. This approach is accepted in the statistical literature, but relatively unknown in the medical/biological research community (15, 16). Briefly, when making multiple comparisons to find the minimum *P* using the log rank test, the false-positive rate (i.e., the percentage of times a marker that has no true prognostic value will be found to have a *P* < 0.05) can approach 40%. Altman's statistical adjustment generates a minimum *P* corrected to yield a true false-positive rate of 5%. The corrected *P* (*P*_{cor}) is calculated as follows: $P_{cor} = \phi(\zeta) [\zeta - (1/\zeta)] \log[e] [(1 - \epsilon) <2> / \epsilon <2>] + 4 \phi(\zeta) / \zeta$, where ζ indicates the probability density function. *P*_{min} is the minimum *P* generated by evaluating multiple cutpoints, ζ is the $(1 - P_{min} / 2)$ quantile of the standard normal distribution, and ϵ denotes the proportion of values excluded from consideration as an optimal cutpoint. Our calculations were done using an ϵ of 0.10. Disease-free survival and local recurrence were subsequently assessed by Kaplan-Meier analysis with log rank for determining statistical significance. All survival analyses were done at 5-year cutoffs. Relative risk was assessed by the univariate and multivariate Cox proportional hazards model. Comparison of β-catenin expression in normal tissue to tumor tissue (high and low expression groups) was made by Wilcoxon rank-sums test with Bonferroni corrections for multiple comparisons. Correlation of β-catenin AQUA score with cyclin D1 AQUA score was made by Spearman correlation. Comparison of β-catenin expression class with the clinical and pathologic variables gender, TNM stage, histologic grade, treatment method (primary EBRT versus primary surgical excision plus radiotherapy), chemotherapy treatment, and oropharyngeal subsite were made using χ^2 analysis. Comparison of β-catenin expression and patient age was made by Spearman correlation. All calculations and analyses were two-tailed where appropriate and done with SPSS 11.5 for Windows (SPSS, Inc., Chicago IL).

Results

Patient demographics. There were 94 patients with primary oropharyngeal carcinoma that met inclusion criteria for AQUA analysis. Mean follow-up time for survivors was 44.9 months and for the entire cohort was 35 months. Seventy-four were male and 20 female, with age ranging from 41 to 79 years old. Eleven patients (11.7%) were TNM stage II, 27 (28.7%) stage III, and 56 (59.6%) stage IV. Oropharyngeal subsites included 40 (42.6%) tonsillar fossae, 49 (52.1%) base of tongue, 3 (3.2%) other oropharynx, and 2 (2.1%) not recorded. Fifty-seven (60.6%) patients were managed with primary EBRT, 35 (37.2%) with surgical excision followed by postoperative EBRT, and 2 (2.1%) not recorded. Twenty patients (21.3%) were treated with chemotherapy. For histologic grade, 6 (6.4%) tumors were well differentiated, 44 (46.8%) were moderately differentiated, 33 (35.1%) were poorly differentiated, and 11 (11.5%) not recorded. Demographic and clinicopathologic variables for the cohort are summarized in Table 1.

Table 1. Demographic, clinical, and pathologic data

	<i>n</i>	<i>β</i> -catenin expression class*		<i>P</i> [†]
		Low	High	
Age, 41-79 y (mean 60.4 y)	94	12	63	0.22
Gender				
Male	74	9	50	0.71
Female	20	3	13	
Site				
Tonsils	40	5	27	0.72
Base of tongue	49	6	32	
Other/not recorded	5	1	4	
TNM stage				
II	11	2	7	0.040 [‡]
III	27	7	15	
IV	56	3	41	
Grade				
Well differentiated	6	2	2	0.18
Moderately differentiated	44		6	31
Poorly differentiated	33	3	21	
Not recorded	11	1	9	
Management				
Primary radiotherapy	57	10	36	0.19
Postoperative radiotherapy	35	2	25	
Not recorded	2	0	2	
Chemotherapy	20	3	12	0.70
*No chemotherapy	74	9	51	

* Does not include 19 cases for which β-catenin expression could not be determined due to insufficient tissue representation in the tissue microarray.

† Does not include patients for whom this information was not available.

‡ Significant at the 0.05 level.

Quantitative immunohistochemistry for β-catenin protein expression (automated image acquisition and analysis) and generation of optimal cutpoint by X-Tile analysis. Of the 94 patients included in this study, 75 (80%) had sufficient tissue for analysis of β-catenin protein expression by AQUA. Tissues deemed insufficient had <10% tumor mask within the histospot, as represented on the tissue microarray. As visualized by fluorescent immunohistochemistry, β-catenin displayed strong membranous staining in a majority of tumors as well as Caski cell line controls (Fig. 1A-C). Normalized AQUA scores were represented on a 1 to 255 scale. β-catenin expression followed a skewed distribution as expected for a cancer tissue biomarker (Fig. 1D). Using the X-Tile program, an optimal cutpoint for β-catenin expression was determined at 45.4 AQUA units, with a Monte Carlo *P* of 0.0430 as determined by X-Tile. Patients with β-catenin expression <45.4 were classified as low expressers (*n* = 12), and patients with β-catenin expression >45.4 were classified as high expressers (*n* = 63). Comparison of clinicopathologic variables with β-catenin expression class showed that patients with high TNM stage were more likely to have high expression of β-catenin (*P* = 0.040). There were no

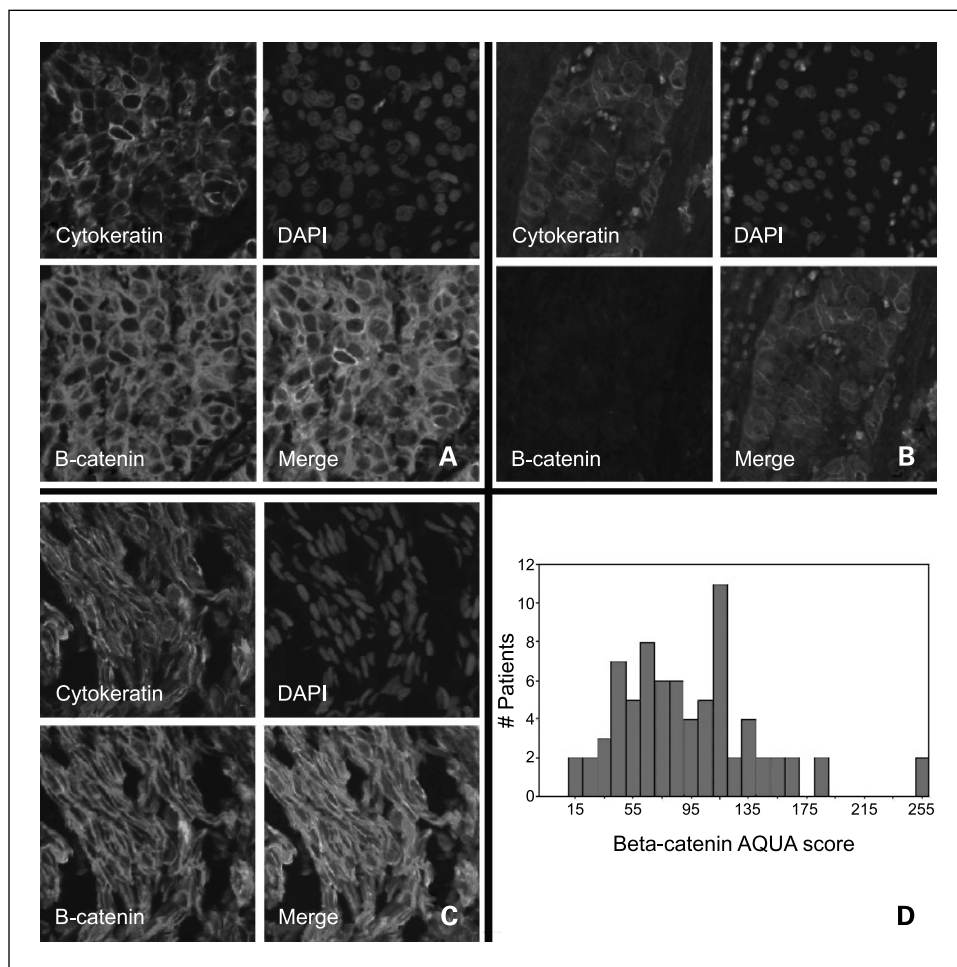


Fig. 1. β -catenin immunofluorescence and AQUA analysis. A, the majority of tumors displayed strong membranous β -catenin expression. B, some tumors displayed loss of β -catenin expression. C, positive control Caski cells also displayed strong membranous staining. Nuclear staining was not predominately observed in any of the tumor samples examined. D, AQUA analysis showed a right skewed distribution for β -catenin expression, consistent with expectations for a biological marker.

other associations between β -catenin expression and clinicopathologic variables. These results are summarized in Table 1.

Quantitative immunohistochemistry (automated image acquisition and analysis) of cyclin D1 expression. The cyclin D1 expression in this cohort was previously reported (11). Briefly, 66% of tissue spots had sufficient tissue for analysis of cyclin D1 expression by AQUA. Cyclin D1 displayed a primarily nuclear expression pattern by immunofluorescence analysis, and normalized AQUA scores were reported on a 1 to 255 scale.

Comparison of β -catenin expression in tumor versus normal tissue. For normal squamous epithelium, 27 of 53 (51%) tissue spots were interpretable for β -catenin expression by AQUA analysis. The mean β -catenin expression in normal squamous epithelium was 77.0 AQUA units (95% confidence interval, 65-89). Comparison of β -catenin showed that tumors in the low expression group had markedly decreased β -catenin expression compared with normal, with a mean of 33.8 AQUA units ($P = 0.001$). Tumors in the high β -catenin group showed a slight but significant gain of expression compared with normal epithelium with a mean of 104.6 AQUA units ($P = 0.006$). These results are summarized in Table 2.

Correlation of β -catenin and cyclin D1 expression by automated image acquisition and analysis. The association between β -catenin and cyclin D1 expression (as determined by AQUA) was analyzed by Spearman correlation. A

significant inverse relationship was found where tumors with high β -catenin had low cyclin D1 and vice versa (Spearman $\rho = -0.351$, $P = 0.007$).

Univariate survival analysis

Local recurrence. The expression status of β -catenin was evaluated for association with local recurrence using Kaplan-Meier survival analysis with log-rank statistic for determining significance. This analysis (Fig. 2A) showed that low β -catenin expression is associated with increased 5-year local recurrence rates. Patients with low β -catenin expression had a local recurrence rate of 79% compared with 29% for patients with high β -catenin tumors ($P = 0.0021$). Univariate Cox regression revealed a hazard ratio for low β -catenin tumors of 3.6 ($P = 0.004$). As use of an optimized cutpoint can result in increased type I error, the Miller-Siegmund correction method was applied to all Kaplan-Meier analyses ($P = 0.0484$).

Disease-free survival. The expression status of β -catenin was also evaluated for association with disease-free survival. Kaplan-Meier analysis (Fig. 2B) showed that patients with low β -catenin-expressing tumors trended toward worse 5-year disease-free survival, but this was not significant after Miller-Siegmund correction ($P = 0.06$, Miller-Siegmund $P = 0.58$). Univariate Cox regression revealed a trend toward increased risk for patients with high β -catenin tumors, but this also failed

Table 2. Expression of β -catenin in normal versus neoplastic tissue

	<i>n</i>	Mean AQUA score	95% CI	<i>P</i>
Normal squamous epithelium	27	82.24	70.4-94.1	
All tumors	75	93.28	82.2-104.3	0.34
Low β -catenin tumors	12	33.80	27.71-39.98	0.002
High β -catenin tumors	63	104.61	93.6-115.6	0.036

NOTE: *P* values are for comparison to normal squamous epithelium after Bonferroni correction.
Abbreviation: 95% CI, 95% confidence interval.

to reach statistical significance (hazard ratio = 1.9, *P* = 0.068). Results of univariate survival analysis are summarized in Table 3.

Multivariate survival analysis. Using the Cox proportional hazards model, we did multivariate analysis to assess the independent predictive value of β -catenin expression for 5-year disease-free survival and local recurrence. The following prognostic variables were also included: management (primary EBRT versus surgery plus EBRT), TNM stage, and histologic grade. For local recurrence, only β -catenin expression status was an independent prognostic factor (*P* = 0.044). For disease-free survival, no independent prognostic factor was found. Results for multivariate survival analysis are summarized in Table 4.

Mutational analysis of head and neck squamous cell carcinoma. Sixty cases of head and neck squamous cell carcinoma were analyzed for both β -catenin and plakoglobin mutation status at key regulatory serine/threonine residues. There were no mutations identified in either β -catenin or plakoglobin following single-stranded conformational polymorphism and direct sequencing analysis.

Discussion

In this study, we aimed to explore the role of β -catenin in the pathogenesis of HNSCC. First we did mutational analysis in a cohort of patients with HNSCC and we found the absence of activating mutations. Subsequently, we analyzed a tissue microarray composed of 94 oropharyngeal cancers for the pattern and levels of expression of β -catenin using a method of *in situ* automated quantitative protein analysis. We found that loss of β -catenin is an independent adverse prognostic factor for local recurrence in patients with oropharyngeal cancer. Patients with tumors classified as low β -catenin had a 3.5-fold increased risk of local recurrence compared with the high β -catenin group. β -catenin levels additionally trended toward predicting disease-free survival.

We also compared β -catenin expression in tumors with that in normal tissue to determine if the low β -catenin group represented loss of expression or if the high group represented increased expression. Tumors classified as low β -catenin had markedly lower β -catenin expression when compared with normal squamous epithelium, indicating that loss of β -catenin

expression identifies the patients with worse prognosis, as opposed to gain of β -catenin conferring an improved prognosis. In multivariate analysis, β -catenin expression status remained an independent prognostic factor for local recurrence. Based on immunofluorescence staining, we also found that nuclear β -catenin expression is rare in oropharyngeal cancers. In addition, there was an inverse correlation between β -catenin protein levels and the T-cell factor target protein cyclin D1. During cancer progression, cells disrupt adhesion-mediated regulatory mechanisms by inactivating adherens junctions function via various signaling mechanisms, such as mitogen-activated protein kinase or phosphoinositide 3-kinase activation and changes in the activity of the small GTPases of the Rho family (17, 18). These signaling pathways also result in up-regulation of cyclin D1 and cell cycle progression. Thus, the inverse correlation between β -catenin and cyclin D1 levels can be explained by the opposing effects that the activation of signaling pathways have on the expression of cyclin D1 and β -catenin proteins.

As previously mentioned, subcellular localization of β -catenin determines its role in carcinogenesis. In cell membrane, β -catenin forms a complex with type I cadherins

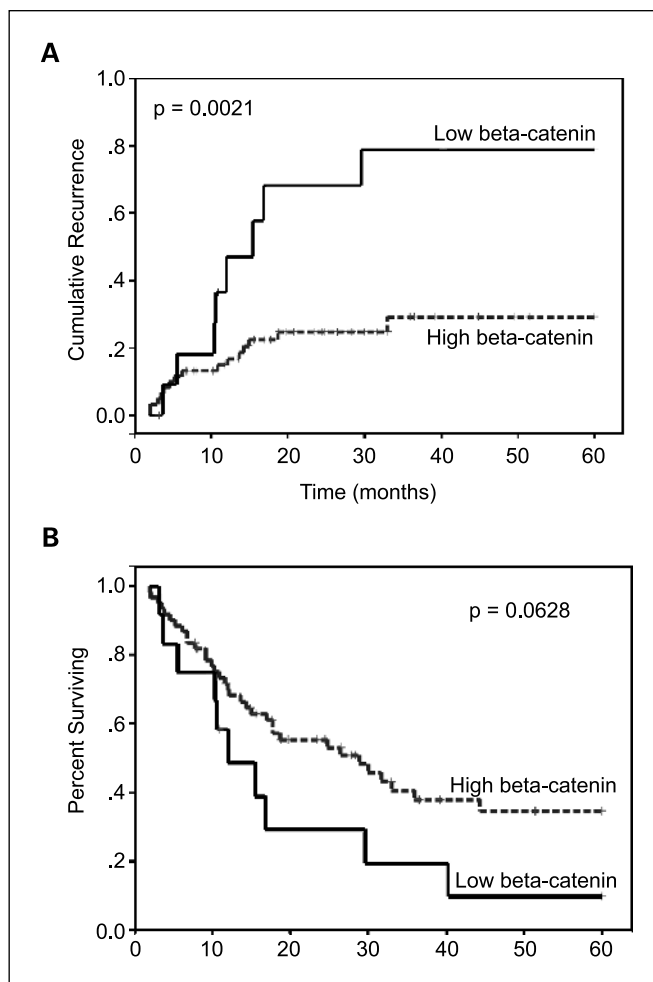


Fig. 2. Kaplan-Meier survival analysis by β -catenin expression. For local recurrence (A), patients in the low β -catenin group showed markedly increased 5-year recurrence rates. For disease-free survival (B), there was a trend toward worse survival in the low β -catenin group, but this did not reach statistical significance.

Table 3. Univariate 5-year survival analysis

	Kaplan Meier analysis			Cox analysis	
	Mean survival (mo)	Cumulative survival or recurrence (95% CI)	P	Hazard ratio (95% CI)	P
Local recurrence					
High β -catenin	23.3	78.8% (53-100)	0.0021*	0.281 (0.1-0.7)	0.004*
Low β -catenin	46.1	29.1% (16-43)		3.561 (1.5-8.4)	
Disease-free survival					
High β -catenin	19.7	9.7% (0-28)	0.0628	0.517 (0.3-1.1)	0.068
Low β -catenin	31.9	34.6% (21-49)		1.935 (0.95-3.9)	

NOTE: All survival analyses were done at 5-year cutoffs.
*Significant at the 0.01 level.

and functions as an adhesion molecule. Reduced expression of membranous β -catenin has been found to be associated with worse outcome in a variety of malignancies including ovarian (19), non-small cell lung cancer (20, 21), melanoma (22), gastric (22), cervical (23), breast (24), bladder (25), and nasopharyngeal carcinoma (26). To the contrary, nuclear (signaling) β -catenin has been correlated to poor outcome in colorectal (11), hepatocellular (27), esophageal (28), hepatoblastoma (29), thyroid (30), and breast (31) carcinomas. β -catenin nuclear accumulation and activation of β -catenin signaling may occur either via loss-of-function mutations in the APC gene, which reduces proteosomal degradation of β -catenin or gain-of-function mutations in β -catenin itself (6). The former mechanism is implicated in colorectal tumorigenesis (32) and the latter in melanomas (33) and hepatocellular carcinomas (34).

In HNSCCs, APC and β -catenin gene mutations are reported to be rare events (35, 36). Nuclear accumulation of β -catenin has also infrequently been reported in HNSCC. To the contrary, many studies have suggested a role for membranous β -catenin in progression and malignant potential of HNSCC. Bankfalvi et al. (37) studied the correlation

of CD44, E-cadherin, and β -catenin levels and prognosis in 93 oral carcinomas, 30 associated metastases, and 12 recurrences using immunohistochemistry. The authors found that decreased β -catenin expression was a predictive marker for nodal metastases. Similar findings were reported by Tanaka et al. (38), who investigated the immunohistochemical expression of β -catenin in 159 oral squamous cell cancers. Gasparoni et al. (39) analyzed the subcellular localization of β -catenin in cultures of human oral normal and malignant keratinocytes and in 24 frozen samples of oral squamous cell carcinomas by a double-staining technique for nucleic acids and β -catenin. The authors found that nuclear β -catenin is a rare finding in oral squamous cell cancers. Loss of β -catenin cell membrane staining correlated with tumor dedifferentiation in laryngeal squamous cell cancers (40). Kudo et al. (41), using an *in vitro* invasion assay, found that reduced expression of membranous β -catenin by immunohistochemistry and Western blot was a frequent event in invasive and metastatic areas of oral SCC. Only one study found significant nuclear localization of β -catenin in HNSCC (42); the authors studied the pattern and expression levels of β -catenin in 88 oropharyngeal and 50 hypopharyngeal tumors by immunohistochemistry and found that nuclear β -catenin expression independently predicted shorter overall survival. In the present study, we found that β -catenin levels in the tumor mask were inversely associated with local recurrence in patients with oropharyngeal squamous cell cancers. We also found absence of gain-of-function mutations in HNSCC.

Taken together, these findings indicate that β -catenin functions predominantly as an adhesion molecule in oropharyngeal squamous cell cancers. The low nuclear levels of β -catenin and the inverse correlation between β -catenin protein levels and T-cell factor target protein cyclin D1 indicate that β -catenin does not function as a signaling molecule in oropharyngeal squamous cell cancers in a manner similar to that described in colon cancer. In oropharyngeal cancer, reduced β -catenin expression most likely destabilizes the cadherin-catenin complex leading to loose cell-cell adherens junctions, cell migration, and metastases. Our study is the only one of its kind that elucidates the role of β -catenin in the

Table 4. Multivariate 5-year survival analysis by Cox regression

Variable	Hazard ratio (95% CI)	P
Local recurrence		
Management	0.428 (0.1-1.2)	0.12
Histologic grade	0.887 (0.4-1.9)	0.76
TNM stage	1.054 (0.5-2.0)	0.87
Low β -catenin	2.841 (0.1-7.7)	0.041*
Disease-free survival		
Management	0.745 (0.4-1.6)	0.44
Histologic grade	0.797 (0.4-1.4)	0.45
TNM stage	1.038 (0.6-1.7)	0.88
Low β -catenin	1.587 (0.7-3.6)	0.28

*Significant at the 0.05 level.

pathogenesis of HNSCC and, more importantly, shows an association between β -catenin levels with oropharyngeal squamous cell cancer prognosis.

The elucidation of function of β -catenin in HNSCC has important therapeutic implications. In addition to their structural-mechanical role, cadherin-catenin-containing adherens junctions play a crucial role in regulating cellular responses

to growth factor-mediated signals. The ability of excessive adherens junctions assembly to decrease cell motility may be associated with the action of adherens junctions as tumor suppressors. Elucidation of the signaling pathways that down-regulate adhesion β -catenin and disrupt adherens junctions in cancer cells will allow the identification of novel targets for inhibiting tumor development.

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