Clinical Significance of Cyclin B1 Protein Expression in Squamous Cell Carcinoma of the Tongue

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ABSTRACT
Purpose: Cyclin B1 plays an important role in control of the G2-M transition of the cell cycle. We have shown recently that overexpression of cyclin B1 is associated with poor outcome in patients with early stage squamous cell carcinoma (SCC) of the lung.

Experimental Design: To determine the role of cyclin B1 in SCC of the tongue, we analyzed tumor specimens from 41 patients with stage II–IV SCC of the tongue who underwent curative surgery using immunohistochemistry.

Results: The median follow-up of all patients was 83 months. Overexpression of cyclin B1 was observed in 15 (37%) of the 41 tumors, a similar frequency to that found in SCC of the lung. Patients whose tumors showed overexpression of cyclin B1 had a poor event-free survival compared with those lacking this feature (P = 0.04 by Log-rank test). Multivariate analysis of traditional clinical/pathological factors showed that cyclin B1 overexpression was an independent prognostic indicator.

Conclusions: Our study indicates that cyclin B1 is overexpressed in a subset of SCC of the tongue and is associated with a more aggressive biological behavior of the disease.

INTRODUCTION
Normal cell proliferation involves a precise sequence of events that is controlled by specific factors, namely cyclins. Cyclins bind to corresponding cyclin-dependent kinases and initiate a complex cascade that regulates the succession and timing of cell cycle phase transition (1). Cyclins A, D, and E regulate the passage from G1 phase to S phase, whereas cyclins A and B direct the transition from G2 phase to M phase (2). Specifically, cyclin B1 and cdc2 are the components of the maturation/mitosis-promoting factor, which plays an important role in G2-M transition (3). Cyclin B1 binds to cdc2, which then becomes dephosphorylated and relocated to the nucleus (4), ensuring the transition toward mitosis. Whereas the cdc2 level is typically constant throughout the cell cycle, cyclin B1 expression is cyclic with a minimal expression in G1 phase, an increased level in S phase, and a peak at the G2-M transition (3, 5). Several factors control the expression and localization of cyclin B1 as pRb/E2F, cyclin A/cyclin-dependent kinase 2, and Plk1 (6, 7). Recent data show that Plk1 acts as a specific kinase phosphorylating cyclin B1 and targets it to the nucleus (7). However, active cyclin B1/cdc2 complex has been detected in the cytoplasm and is implicated in the formation of the mitotic spindle (8). Thus, cyclin B1, through its trafficking between the cytoplasm and the nucleus, seems to play a role in coordinating the mitotic process in both compartments.

Cyclin B1 overexpression has been reported in breast, colon, prostate, and head and neck cancers (9–11, 2). Recently, we reported that cyclin B1 was overexpressed frequently in early stage NSCLC, especially the squamous subtype, and the overexpression was associated with a poor clinical outcome (12). Because cyclin B1 has a direct effect on mitosis, overexpression of cyclin B1 may lead to uncontrolled cell proliferation, which is a characteristic of SCC of the oral cavity (13, 14).

In this study, we investigated the potential role of cyclin B1 in SCC of the tongue, the second most frequently occurring tumor in the oral cavity (15). Tumor specimens from 41 patients with stage II–IV SCC of the tongue were analyzed for cyclin B1 expression using immunohistochemistry, and results were correlated with clinical/pathological factors and patients outcome. Our data indicate that cyclin B1 is overexpressed frequently in SCC of the tongue and that its overexpression is associated with a poor outcome.

MATERIALS AND METHODS
Study Population. This is a retrospective study where oral tongue SCC specimens were obtained from archived tissue samples of surgically resected pathological stage II–IV tumors. All 41 patients chosen indiscriminately were treated at The University of Texas M. D. Anderson Cancer Center (Houston, TX) between 1991 and 1994. All patients underwent surgery and were followed a median of 83 months after surgical treatment. Patients with lymph node involvement or bulky tumors (T3–T4) generally received standard radiotherapy and/or chemotherapy after surgery. Survival data were available for all

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The abbreviations used are: NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma.
patients with a ≥10 months of follow-up. The study population consisted of 25 men and 16 women with mean age of 56.2 years (SD ± 11.4).

**Immunohistochemistry for Cyclin B1 Protein Expression.** Paraffin-embedded, 4-μm-thick tissue sections from all 41 primary tumors were stained for cyclin B1 using a primary mouse monoclonal antibody (NCL-Cyclin B1; NovoCastra, Newcastle, United Kingdom). Slides were baked at 60°C for 1 h and then deparaffinized through a series of xylene baths. The samples were rehydrated in graded alcohols. To retrieve the antigenicity, the tissue sections were treated with microwaves in 10 mM citrate buffer (pH 6.0) three times for 3 min. The sections were then immersed in methanol containing 0.3% hydrogen peroxide for 20 min to block the endogenous peroxidase activity and were incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated overnight at 4°C with primary anticyclin B1 at a 1:15 dilution. The sections were processed using standard avidin-biotin immunohistochemistry according to the manufacturer’s recommendations (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen, and commercial hematoxylin was used for counterstaining. Tissue sections of normal lymph node were used as positive staining controls and were also stained with the primary antibody omitted to confirm staining specificity.

The cyclin B1 labeling index was defined as the percentage of tumor cells displaying cytoplasmic or nuclear immunoreactivity, and it was calculated by counting the number of cyclin B1-stained tumor cells among ≥1000 tumor cells from representative areas of each tissue section. In our previous study of early stage NSCLC (12), we used 15% labeling index as a cutoff point. The same cutoff point was applied in this study. Cells were counted in ≥4 fields (at ×400) in these areas. All slides were scored concomitantly by two investigators (K. A. H. and J. C. S.).

**Statistical Analysis.** Survival curves were estimated by the Kaplan-Meier method, and the resulting curves were compared using the Log-rank test. Fisher’s exact test and the χ² test were used to analyze the association between two categorical variables. P < 0.05 was considered to be statistically significant. Event-free survival accounts for metastasis, recurrence, or death as an event, whereas disease-free survival addresses metastasis, recurrence, or death related to cancer as an event. Immunohistochemical analysis was performed in a blinded manner with respect to the clinical information about the subjects. Multivariate analysis was performed according to the Cox proportional hazards model. Because of the sample size, two covariates were analyzed at a given time. The variables examined were cyclin B1 status compared with disease stage (stage II versus stages III and IV).

**RESULTS**

In histologically normal squamous epithelium adjacent to tumors, cyclin B1 expression was typically undetectable. However, focal areas of positively stained cells could be observed in the basal and parabasal layers in few cases (Fig. 1a); the number of positively stained cells was small (always <1%). In contrast to previous reports of cyclin B1 in the esophagus and the floor of the mouth (16, 2), where cyclin B1 expression was located mainly in the nucleus, cyclin B1 was located mainly in the cytoplasm. In tumor tissues, the staining was generally heterogeneous (Fig. 1b); some tumors showed rare positively stained tumor cells (Fig. 1c), some showed scattered positively stained cells, and others stained uniformly in the majority of tumor cells (Fig. 1e). We also observed that the staining was mainly cytoplasmic and more intense in poorly differentiated areas (Fig. 1f). This showed only a trend of association between differentiation and overexpression of the cyclin B1 but did not reach statistical significance (P = 0.09; Table 1). Interestingly, cytoplasmic staining of the protein was commonly seen in dysplastic areas adjacent to tumors. Among 10 dysplastic lesions identified in different specimens, 3 showed an increase in cyclin B1 expression (>5%) as compared with normal-appearing epithelial areas, suggesting that such alteration might occur early in tumorigenesis in some cases. An example of cyclin B1 expression in dysplastic regions is shown in Fig. 1d.

In our previous study of early stage NSCLC, we used the 15% labeling index as a cutoff point and found that overexpression of cyclin B1 was significantly associated with a poor clinical outcome (12). We applied the same criteria used in our previous lung study for overexpression of cyclin B1. On the basis of these criteria, 15 (37%) of the 41 tongue SCC specimens showed cyclin B1 overexpression, which is comparable with the 34% overexpression detected in the early stage SCC of the lung (12). Negative or low-level expression of cyclin B1 was recorded in 26 (63%) of the tumor specimens. Table 1 summarizes the potential associations between the cyclin B1 expression status and the clinical/pathological parameters. Cyclin B1 expression status did not differ significantly with respect to age, gender, tumor grade, nodal status, or clinical stage.

When cyclin B1 expression status was associated with patients’ clinical outcomes, 9 (60%) of the 15 patients whose tumors showed cyclin B1 overexpression were dead compared with only 9 (35%) of the 26 patients whose tumors lacked cyclin B1 overexpression during a similar duration of follow-up (77.3 months versus 82.9 months, respectively). However, the difference did not reach statistical significance (P = 0.14 by Log-rank test; Fig. 2a). When overall survival, disease-free survival, and event-free survival were analyzed, patients whose tumors showed cyclin B1 overexpression demonstrated a shorter survival duration than patients whose tumors showed no cyclin B1 overexpression; however, this was only statistically significant for event-free survival (P = 0.04 by Log-rank test; Fig. 2b). Of the 15 patients whose tumors overexpressed cyclin B1, 11 (73%) experienced an event (i.e., metastasis, recurrence, or death) versus 10 (39%) of the 26 patients whose tumors showed no cyclin B1 overexpression. The mean 5 years overall and event-free survival rate for patients whose tumors showed cyclin B1 overexpression was 47% as compared with 68% in patients whose tumors showed no cyclin B1 overexpression. As expected, nodal status (N₀ versus N₁₋₃, respectively) and clinical stage were also associated with a poorer overall survival (P = 0.02 and P = 0.05, respectively). In a multivariate analysis using cyclin B1 expression, nodal status, and clinical stage as parameters, cyclin B1 remained an independent prognostic factor for event-free survival (P = 0.03). Because of the relatively small sample size and incomplete smoking and alcohol intake data, the associations between cyclin B1 expression and these parameters are not provided.
DISCUSSION

Uncontrolled cell proliferation is a key feature in malignancies and is often associated with prognosis. In normal conditions, the integrity of the genetic material during the different phases of the cell cycle is strictly monitored, and proliferation is firmly regulated (17). Although most of the studies have focused on the controlled G1 to S transition phase, which is frequently altered in tumorigenesis (18), the control of subsequent stages of

Fig. 1 Immunohistochemical staining patterns of cyclin B1 in tongue SCC. a, normal stratified squamous epithelium with cyclin B1 staining in the basal and parabasal layers (×400). b, heterogeneous staining of tumor tissue (×100). c, predominantly negative staining in carcinoma cells with few positive cells (×200). d, mild epithelial dysplasia showing increased staining in the basal and parabasal cells (×200). e, uniform staining in most of the tumor cells (×200). f, prominent staining of the less differentiated areas of the tumor nest (×200).
the cell cycle is also important but less investigated. For example, cyclin B1/cdc2 plays an important role in G2-M transition (3) where a deregulation of this complex may promote cell proliferation which in turn forces the cell toward mitosis. Furthermore, recent findings indicate that activation of cyclin B1 and its associated cdc2 kinase can override a p53-mediated G2-M phase checkpoint (24). Thus, overexpression of cyclin B1, either by increased synthesis or impaired degradation, or its improper localization because of failure of the nuclear/cytoplasmic homeostasis may play a decisive role in cell proliferation.

Interestingly, we observed a predominant cyclin B1 expression in the cytoplasm of tumor cells rather than in the nucleus. The significance of this phenomenon is unclear, but one explanation could be that nuclear localization of cyclin B1 is transient, whereas cytoplasmic accumulation is continuous throughout the cycle in premalignant and malignant cells. Another explanation may be that anticyclin B1 antibody has a higher affinity to cytoplasmic cyclin B1 because of epitopic modifications. However, the presence of concomitant nuclear staining does not support this explanation. It has been reported that cytoplasmic cyclin B1 colocalizes with microtubules during progression through G2 phase (5), and its interaction with the cytoskeleton is mediated through microtubule-associated protein kinase 4 (Ref. 25). In addition, cyclin B1/cdc2 complex is active in the cytoplasm and is involved in establishing the mitotic spindle preceding the mitotic nuclear events (8). Another study reported that cytoplasmic-located cyclin B1 was able to induce mitosis, although to a much lower extent than nuclear cyclin B1 (24). Thus, a notable overexpression of cytoplasmic cyclin B1 may push the cell toward mitosis. It is possible that the overexpressed cytoplasmic cyclin B1 interacts with other cytoplasmic proteins involved in pathways that promote malignant transformation, or it accumulates to a level that may saturate its carrier and remain in the nucleus to induce mitosis. This intriguing phenomenon requires further investigation to address it because that is beyond the scope of the current study. Nevertheless, cytoplasmic overexpression of cyclin B1 may provide a therapeutic target, because abnormal localization of this protein may trigger a cytotoxic immune response and allow the development of potential immunotherapy to target such cells.

In this study, we found that cyclin B1 is overexpressed in a significant number of SCCs of the tongue. Of the tumors obtained from 41 patients, 37% showed overexpression of cyclin B1. Interestingly, in the lung cancer study performed previously by our group, cyclin B1 overexpression was observed at a similar frequency (34%) in the SCC subtype, whereas overexpression of cyclin B1 is relatively rare in non-SCC subtypes, such as adenocarcinoma (12). Murakami et al. (16) studied 87 esophageal SCC and found that cyclin B1 was overexpressed in 63 (72%) of the tumors. Such high frequency can be attributed to the much lower cutoff point used in that study (5%). Overall, the data support the notion that cyclin B1 might be used as a biomarker for SCCs.

Important, our data indicated that cyclin B1 staining is detected in both the cytoplasm and the nucleus of some of the normal-appearing epithelium. This is consistent with the observation that cyclin B1/cdc2 complex is constantly shuttled between the cytoplasm and the nucleus and the presence of cyclin B1 nuclear export sequence that prevents its accumulation in the nucleus until needed (19–21). Such accumulation may be achieved through phosphorylation of cyclin B1 at the nuclear export sequence, thereby decreasing its nuclear-export rate (21, 22, 4), allowing the activation of cdc2 and entering cells into mitosis. After the cell commits and starts mitosis, cyclin B1 is degraded through ubiquitination rendering cdc2 inactive (23). Unscheduled or continuous overexpression of cyclin B1 throughout the cell cycle will lead to the activation of the already existing cdc2, which in turn forces the cell toward mitosis. Furthermore, recent

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a Mean age, 56.2 years.

b The histological grade of one tumor specimen was baseloid type and, therefore, not included in the analysis.

c The nodal status of one patient was indeterminate and, therefore, not included in the analysis.

In this study, we noted that cyclin B1 staining is detected in both the cytoplasm and the nucleus of some of the normal-appearing epithelium. This is consistent with the observation that cyclin B1/cdc2 complex is constantly shuttled between the cytoplasm and the nucleus and the presence of cyclin B1 nuclear export sequence that prevents its accumulation in the nucleus until needed (19–21). Such accumulation may be achieved through phosphorylation of cyclin B1 at the nuclear export sequence, thereby decreasing its nuclear-export rate (21, 22, 4), allowing the activation of cdc2 and entering cells into mitosis. After the cell commits and starts mitosis, cyclin B1 is degraded through ubiquitination rendering cdc2 inactive (23). Unscheduled or continuous overexpression of cyclin B1 throughout the cell cycle will lead to the activation of the already existing cdc2, which in turn forces the cell toward mitosis. Furthermore, recent
tongue could be achievable. This prognostic significance was also detected in lung SCC (12) and esophageal SCC (16). Thus, cyclin B1 expression might be a useful prognostic biomarker.

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