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Overexpression of CDK2 Is a Prognostic Indicator of Oral Cancer Progression

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Cyclins and cyclin-dependent kinases (CDKs) play key roles in cell cycle regulation, a process of which dysregulation can lead to uncontrolled cell growth and hence to cancer. We have already reported the alteration of CDK4 and cyclin D1 expression in oral cancer. In this study, we examined by immunohistochemistry the expression of CDK2, and cyclins A and E in 20 normal oral mucosa, 42 dysplastic epithelia, and 103 oral squamous cell carcinomas (SCCs). The expressions of CDK2, and cyclins A and E were not detected in the normal epithelium and significantly altered from epithelial dysplasia to SCC. While there were no significant correlations between the expression of cyclins A, E and the patients' survival, CDK2 expression was significantly correlated with lymph node involvement (P=0.025), tumor differentiation (P=0.032), mode of tumor invasion (P=0.017), and shorter survival period (P=0.0173). These results suggest that the elevated expression of CDK2 is a critical factor in oral cancer progression and can be used as a negative predictive marker of the patients' prognosis.

Key words: CDK2 — Cyclin A — Cyclin E — Oral cancer — Prognosis

Cell proliferation is ultimately dependent on cell cycle control, and the decision to continue to proliferate is made mainly during G1 phase as a result of the activities of G1 cyclins and cyclin-dependent kinases (CDKs) complexes.[1–8] Among those kinases that regulate G1 progression, CDK4 and CDK6 are activated by association with cyclin D in the mid G1.[9,10] CDK2 is activated by binding to cyclin E, and its activity is essential for transition through the restriction point in the late G1.[7,8,11,12] Subsequently, cyclin A is expressed and is thought to be required, in association with CDK2, for progression through the S phase.[13] Amplification and/or overexpression of cyclin E has been reported in colorectal, breast, lung, ovarian and uterus carcinomas.[14–22] Overexpression of cyclin A has been reported in lung[20] and uterus carcinomas.[22] In the head and neck, amplification of cyclin D in oral squamous cell carcinoma (SCC) has recently been reported.[23,24] There are few reports on CDK2, and cyclins A and E in oral SCC, and the molecular events that underlie the histological progression are not well understood. In this study, we focused our attention on the G1/S and S to G2 transitions in the cell cycle and examined by immunohistochemistry the expression of CDK2, as well as its regulatory partners, cyclins A and E, in oral premalignant lesions and cancers. We also performed western blot analysis in matched sets of tumors and normal tissues. Furthermore, we analyzed the relationship between the expressions of CDK2 and cyclins A and E, and the clinicopathological parameters and patients' survival outcome.

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MATERIALS AND METHODS

Patients Tissue samples of 20 normal oral mucosa specimens, 42 epithelial dysplasia specimens, and 103 oral SCC patients were investigated. The patients underwent surgery at the Department of Oral and Maxillofacial Surgery II, Okayama University Dental School (Okayama) from April 1989 to May 1998 and their clinical follow-up data were available. All patients had received no prior therapy, such as chemotherapy or radiotherapy, before surgery. The patients were followed up until death or till the end of 1998. The samples were obtained at the time of biopsy for the initial diagnosis and at the time of surgery, and were routinely processed. They were formalin-fixed and paraffin-embedded for the immunohistochemical study or immediately frozen and kept at −80°C for the western blot analysis. The tumors were clinically staged according to TMN system.[25] The grade of tumor differentiation was determined according to the criteria of WHO histological grading.[26] The modes of tumor invasion were classified according to Jacobson's classification.[27]

Immunohistochemistry Representative blocks of formalin-fixed, paraffin-embedded tissues were cut at 4 μm thickness. The sections were deparaffinized with xylene, rehydrated in graded alcohol and microwaved for 15 min in citrate buffer (pH=6.0). Three-percent hydrogen peroxide was then applied to block the endogenous peroxidase activity. The sections were then further blocked with 5% dry milk, and the primary antibodies were applied as follows: polyclonal antibody to CDK2 (Santa Cruz Biotechnology, Santa Cruz, CA) was applied at a dilution of 1:300; polyclonal antibody to cyclin A (Santa Cruz Bio-

technology) was applied at a dilution of 1:70; monoclonal antibody to cyclin E (Pharmingen, San Diego, CA) was applied at a dilution of 1:100; and monoclonal antibody to proliferating cell nuclear antigen (PCNA) (Novocastra, Newcastle, UK) was applied at a dilution of 1:300. Immunostaining was performed with the Envision system (DAKO, Carpinteria, CA) according to the manufacturer's instructions. The peroxidase activity was visualized by applying diaminobenzidine chromogen, containing 0.05% hydrogen peroxidase. The sections were then counterstained with methylgreen, dehydrated, cleared, and mounted. Negative control staining was carried out by substituting non-immune mouse or rabbit serum for the primary antibodies.

Immunohistochemical assessment Immunostaining was evaluated in a coded manner without knowledge of the clinical and pathological parameters by two independent observers (M. M. and S. S.). For each section, 10 high-power fields were chosen, and a total of at least 1000 cells was evaluated. The results were expressed as the percentage of positive cells counted. To confirm the reproducibility, 25% of the slides were chosen randomly and scored twice. All duplicates were similarly evaluated. The percentage of positive cells in each case was semiquantitatively evaluated into one of the following five groups: (a) immunoreactivity completely absent (negative, 0%); (b) <5%; (c) <30%; (d) <50%; and (e) >50%. In the present study, cases showing >5% of positive cells were defined as "positive" as previously reported.[28, 29]

Western blot analysis Assessment of the expression levels of CDK2, and cyclins A and E by western blotting was performed on 2 normal epithelia, 4 severe epithelial dysplasia and 6 oral SCCs. The proteins were extracted from the tissues and subjected to western blot analysis with the same specific antibodies used in the immunohistochemical staining studies. The tissues were snap-frozen in liquid nitrogen and stored at −80°C. The samples were homogenized in lysis buffer (150 m*M* NaCl, 1% NP-40, 1% sodium deoxycholate, 2% sodium dodecyl sulfate (SDS), 50 m*M* Tris-HCl (pH 8.0)) with protease inhibitors (50 m*M* sodium fluoride, 200 m*M* sodium orthovanadate, 2 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride and 1 μM clast-lactacystin β-lactone) at 4°C. The homogenates were incubated on ice for 30 min and then centrifuged. The supernatants were assayed for protein content by the Bio-Rad DC Kit (Bio-Rad, Munich, Germany) and boiled for 5 min in Laemmli loading buffer (62.5 m*M* Tris-HCl (pH 6.8), 2% SDS, 0.2 *M* dithiothreitol (DTT), 20% glycerol and 0.001% bromophenol blue) at 95°C. Fifty micrograms of protein from each sample was electrophoresed in a 12% SDS-polyacrylamide gel and then electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was incubated with each of the following antibodies, anti-CDK2 (diluted 1:2500), cyclin A (diluted 1:1000), cyclin E (diluted 1:1000), and actin (diluted 1:1000) for 16 h at 4°C. Horseradish peroxidase-conjugated rabbit anti-mouse antibody and goat anti-rabbit antibody were used as secondary antibodies, and the bands were visualized using the Amersham enhanced chemiluminescence (ECL) nonradioactive method according to the manufacturer's instructions (Amersham Buchler, Braunschweig, Germany).

Statistical analysis The correlations between the discrete variables were assessed using the χ^2 and the Fisher exact tests. The mean values were compared using the Mann-Whitney test (for two categories). The time to treatment failure and its dependence on putative prognostic factors were investigated by the log-rank method and Cox's proportional hazard regression analysis.[30] Kaplan-Meier curves were constructed to show the probability of the patients' survival as a function of time after diagnosis.

RESULTS

Clinical and pathological cohort data Regarding the characteristics of the 103 oral SCC patients, there were 64 males (62.1%) and 39 females (37.9%), with an overall mean age of 66.1 years (range: 23–91 years). Regarding grading, 42 (40.8%) of the tumors were classified as grade I, 39 (37.9%) as grade II, and 22 (21.3%) as grade III. Concerning the clinical stages, there were 16 cases (15.5%) in T1, 48 cases (46.6%) in T2, 13 cases (12.6%) in T3, and 26 cases (25.3%) in T4. As for the lymph node involvement, 74 cases (71.8%) were N0, 8 cases (7.8%) were N1, 20 cases (19.4%) were N2, and 1 case (1.0%) was N3. There were 16 cases (15.5%) in stage I, 31 cases (30.1%) in stage II, 18 cases (17.5%) in stage III, and 38 cases (36.9%) in stage IV.

Immunohistochemical staining patterns of the normal epithelia, dysplasia and oral cancers In the normal epithelia, the expressions of CDK2 and cyclin E were not detectable. The expression of cyclin A was detected in the basal cell layer (<5% positive cells) (Table I, Fig. 1).

In the mild and moderate dysplasia, cyclins A and E exhibited infrequent immunoreactivity. In the severe dysplasia, 3 cases (21.4%) were positive for CDK2, 7 cases (50%) were positive for cyclin A, and 8 cases (57.1%) were positive for cyclin E (Table I). The expression of CDK2 was observed sporadically in the basal cell layer, and cells positive for cyclin A were seen predominantly in the basal or parabasal cell layer. Regarding the expression of cyclin E, positive cells were observed in the prickle cell or differentiated cell layer (Fig. 2).

Positive immunohistochemical staining for CDK2, cyclin A, and cyclin E was detected in the nuclei of the tumor cells (Fig. 3). The proportions of positively stained tumor cells were as follows: 0.1–35.5% of the cells were positive for CDK2; 0–33.3% were positive for cyclin A;

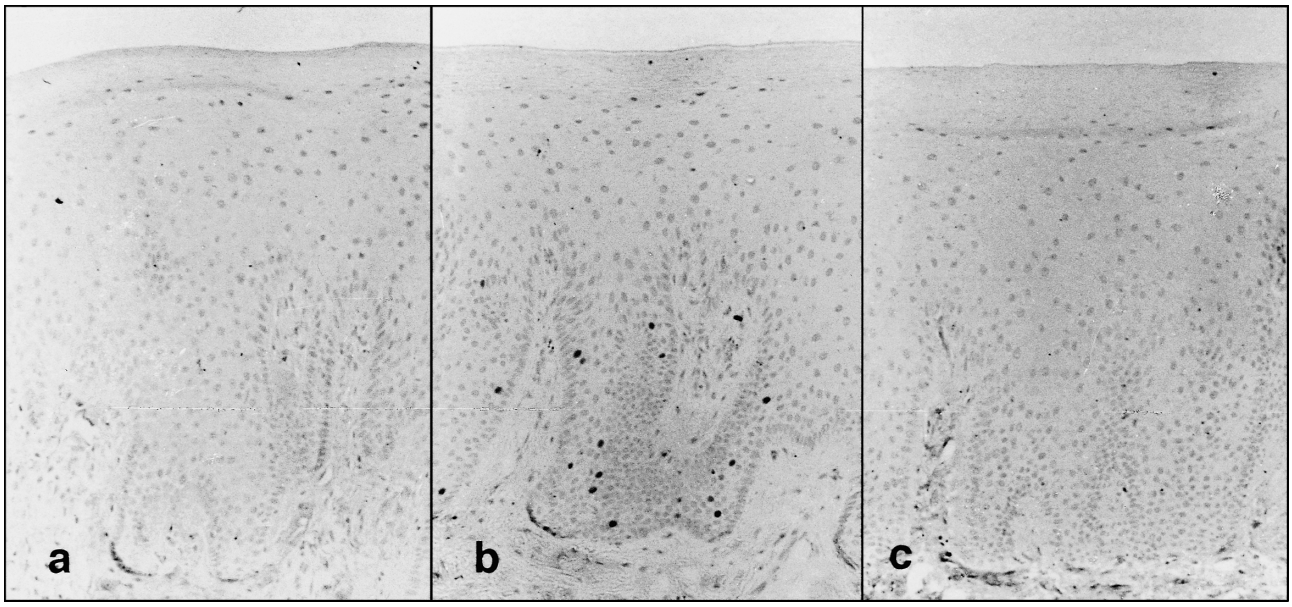

Fig. 1. Immunohistochemical staining patterns in normal epithelia. In the normal epithelium, cells positive for CDK2 (a) and cyclin E (c) were not detected. A few positive cells for cyclin A (b) were observed sporadically in the basal cell layer (<5% positive cells). (×100).

Table I. Expression of CDK2, Cyclin A, and Cyclin E in Normal, Premalignant, and Oral Cancer

	Normal (%) (n=20)	Premalignant (leukoplakia)			SCC (%) (n=103)
		Mild (%) (n=12)	Moderate (%) (n=16)	Severe (%) (n=14)	
CDK2	0 (0)	0 (0)	0 (0)	3 (21.4)	64 (62.1)
Cyclin A	0 (0)	0 (0)	2 (12.5)	7 (50)	80 (77.7)
Cyclin E	0 (0)	0 (0)	1 (6.25)	8 (57.1)	65 (63.1)

and 0–26.8% were positive for cyclin E. In the present study, cases showing >5% of positive cells were defined as "positive," as previously reported.[28, 29] So, CDK2 positivity was detected in 64 of 103 cases (62.1%), cyclin A positivity was observed in 80 (77.7%), and cyclin E positivity was detected in 65 (63.1%) (Table I). The overall results of the immunohistochemical analysis are summarized in Table I. The expression of CDK2 was observed predominantly in the basal areas of tumor nests and in the invading edges, and the staining of cyclin A was similar to that of CDK2. Regarding the expression of cyclin E, positive cells were observed among tumor cells that existed in the differentiated areas (Fig. 3).

Correlation between the expressions of CDK2, cyclins A and E and proliferative rate The PCNA labeling index of the tumors which were positive for CDK2 (24.2±12.2%) and cyclin A (21.8±12.8) was significantly higher than that of the tumors which were negative for CDK2 (14.3±10.7%) and cyclin A (14.3±11.2). However, although in general the PCNA labeling index of the tumors which were positive for cyclin E (21.5±11.7%) was higher than that of the tumors which were negative for cyclin E (17.8±14.1%), the difference was not statistically significant.

Western blot analysis To confirm the results observed by immunohistochemical staining, western blot analysis was performed using lysate obtained from the tissue samples. The expression levels of CDK2, cyclins A and E detected by immunoblotting correlated well with the findings revealed by immunohistochemistry (Fig. 4).

Correlation of CDK2 and cyclins A and E immunostaining with patients' clinicopathological parameters and survival Correlative analyses were performed between CDK2 and cyclins A and E immunostaining and

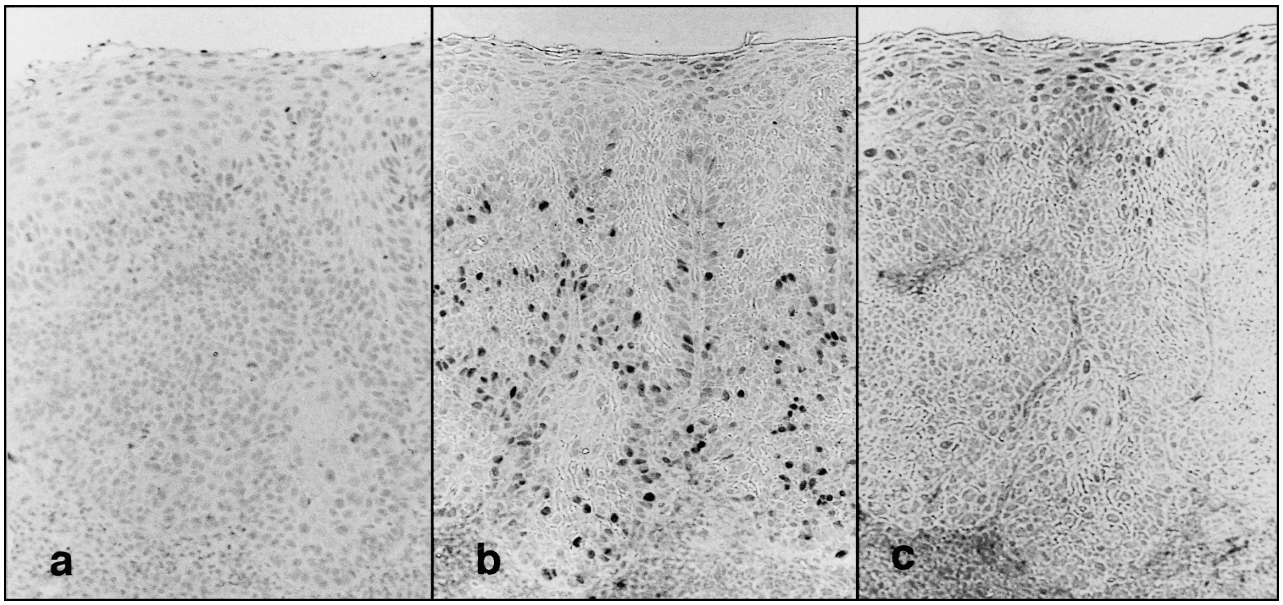

Fig. 2. Immunohistochemical staining patterns in epithelial dysplasia. In epithelial dysplasia, the expression of CDK2 was observed sporadically in the basal cell layer (a). Positive cells for cyclin A were seen predominantly in the basal or parabasal cell layer (b). Cyclin E-positive cells were predominantly observed in the prickle cell or differentiated cell layer (c). (×100). Immunohistochemical staining was performed with an Envision system and 3,3′-diaminobenzidine development. Cells were counterstained with methyl green.

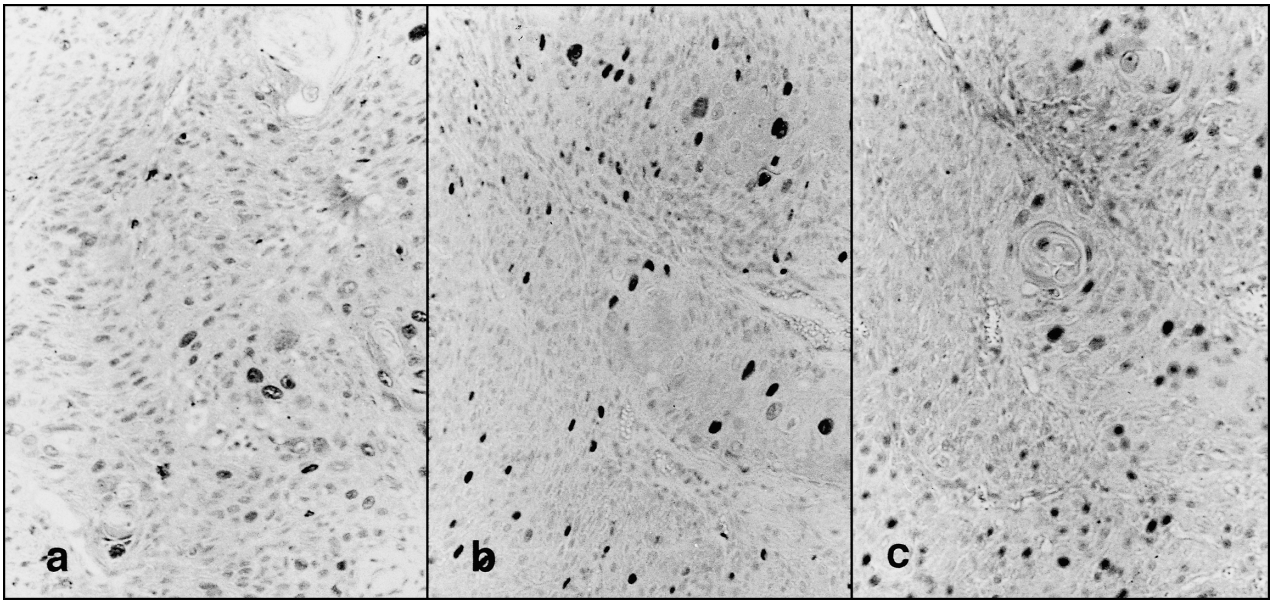

Fig. 3. Staining patterns in oral squamous cell carcinoma. In oral squamous cell carcinoma, the expressions of CDK2 (a), cyclin A (b), and cyclin E (c) were detected in the nuclei of tumor cells. (×200). Immunohistochemical staining was performed with an Envision system and 3,3′-diaminobenzidine development. Cells were counterstained with methyl green.

various clinicopathological parameters. CDK2 expression closely correlated with the mode of tumor invasion (P=0.017), tumor differentiation (P=0.032), and lymph node involvement (P=0.025, Table II). Cyclin A expression correlated significantly with the mode of tumor invasion (Table II). For cyclin E, however, there was no

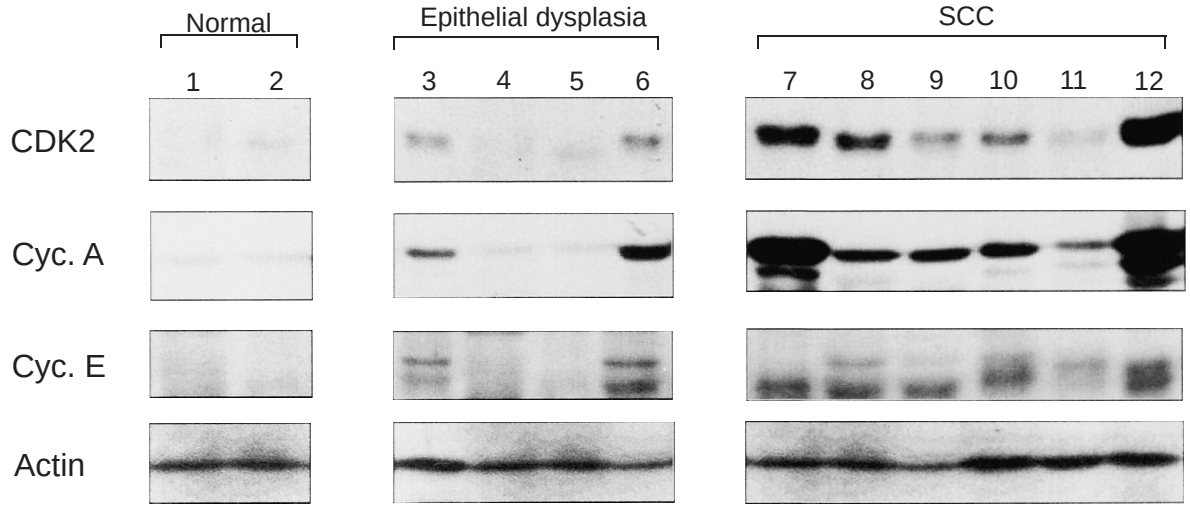

Fig. 4. Western blot of CDK2, cyclin A, and cyclin E in oral tissues. Western blot analysis of CDK2, cyclin A, and cyclin E proteins was performed on total protein extracts obtained from normal epithelia samples (lanes 1, 2), from severe epithelial dysplasia samples (lanes 3, 4, 5, 6), and from oral SCC samples (lanes 7, 8, 9, 10, 11, 12). Note that each western blot analysis was performed on the same lysate (50 μg of protein) and actin antibodies were used to confirm equal loading. For additional details, see "Materials and Methods."

Table II. Expression of CDK2, Cyclin A, and Cyclin E and Clinicopathological Parameters

		CDK2			Cyclin A			Cyclin E		
		Positive (%) (n=64)	Negative (%) (n=39)		Positive (%) (n=80)	Negative (%) (n=23)		Positive (%) (n=65)	Negative (%) (n=38)	
Age (yr)	<60	20 (69.0)	9 (31.0)	P=0.371	22 (75.9)	7 (24.1)	P=0.783	20 (69.0)	9 (31.0)	P=0.441
	≥60	44 (59.4)	30 (40.6)		58 (78.4)	16 (21.6)		45 (60.8)	29 (39.2)	
Sex	Male	39 (60.9)	25 (39.1)	P=0.112	46 (68.8)	18 (31.2)	P=0.070	38 (59.4)	26 (40.6)	P=0.315
	Female	25 (64.1)	14 (35.9)		34 (87.2)	5 (12.8)		27 (69.2)	12 (30.8)	
Clinical T stage[a)]										
	T1 and T2	39 (60.9)	25 (39.1)	P=0.748	51 (79.7)	13 (20.3)	P=0.529	43 (67.2)	21 (32.8)	P=0.877
	T3 and T4	25 (64.1)	14 (35.9)		29 (74.4)	10 (25.6)		22 (56.4)	17 (43.6)	
Lymph node involvement[a)]										
	Yes (N1, N2, N3)	23 (79.3)	6 (20.7)	P=0.025	24 (82.8)	5 (17.2)	P=0.438	16 (55.2)	13 (44.8)	P=0.296
	No (N0)	41 (55.4)	33 (45.6)		56 (75.7)	18 (24.3)		49 (66.2)	25 (37.8)	
Tumor differentiation[b)]										
	Well/Moderate	46 (56.8)	35 (43.2)	P=0.032	64 (79.0)	17 (21.0)	P=0.530	54 (66.7)	27 (33.3)	P=0.151
	Poorly	18 (81.8)	4 (18..2)		16 (72.7)	6 (27.3)		11 (50.0)	11 (50.0)	
Mode of tumor invasion[c)]										
	Grade 1/2	8 (40.0)	12 (60.0)	P=0.017	11 (55.0)	9 (45.0)	P=0.011	12 (60.0)	8 (40.0)	P=0.927
	Grade 3/4	57 (68.7)	26 (31.3)		70 (84.3)	13 (15.7)		51 (61.4)	32 (38.6)	

a) UICC, TNM classification of malignant tumor (1997).
b) WHO histological typing of oral and oropharyngeal tumors (1971).
c) Jacobsson, P. A. *et al*.

significant correlation with any parameter (Table II). The staining results of CDK2, cyclins A and E were also evaluated for their correlation with the patients' survival status. CDK2-positive oral cancer revealed a significantly lower survival outcome than CDK2-negative oral cancer (P=0.0173, Fig. 5). For cyclins A and E staining, however, no significant association was found with the patients' survival (P=0.0784, P=0.2938, respectively, Fig. 5). It is known that CDK2 may function as a complex with cyclin A or E.[7, 8, 11–13)] We determined whether more imformation

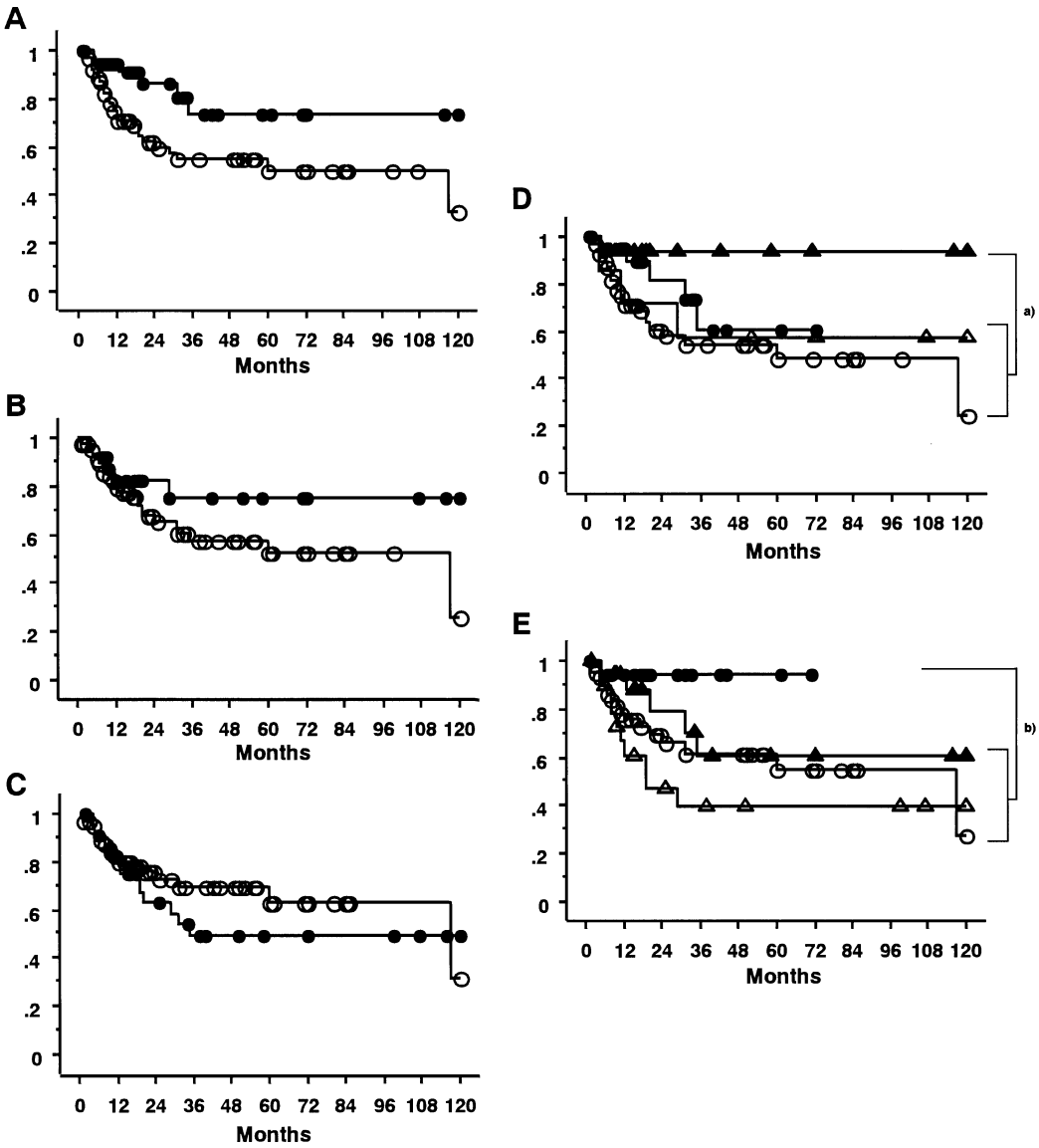

Fig. 5. Kaplan-Meier curve of time to treatment failure (n=103). Cumulative Kaplan-Meier survival curve in a series of 103 patients stratified according to the immunohistochemical expression of CDK2, cyclin A, and cyclin E. +, positive; −, negative. (A) Correlation with CDK2 (+) and CDK2 (−). ○, CDK2 (+)=62.1% (n=64); ●, CDK2 (−)=37.9% (n=39), and P=0.0173. (B) Correlation with cyclin A (+) and cyclin A (−). ○, cyclin A (+)=77.7% (n=80); ●, cyclin A (−)=22.3% (n=23), and P=0.0784. (C) Correlation with cyclin E (+) and cyclin E (−). ○, cyclin E (+)=63.1% (n=65); ●, cyclin E (−)=36.9% (n=38), and P=0.2936. (D) Kaplan-Meier curve for overall survival comparing patients according to their CDK2 and cyclin A status. Patients were assigned to four subgroups according to the percentage of positive tumor cells for each of the two antigens: ○, CDK2 (+), cyclin A (+), n=57; △, CDK2 (+), cyclin A (−), n=7; ●, CDK2 (−), cyclin A (+), n=23; ▲, CDK2 (−), cyclin A (−), n=16.[a)] P=0.0392. (E) Kaplan-Meier curve for overall survival, comparing patients according to their CDK2 and cyclin E status. ○, CDK2 (+), cyclin E (+), n=45; △, CDK2 (+), cyclin E (−), n=19; ●, CDK2 (−), cyclin E (+), n=20; ▲, CDK2 (−), cyclin E (−), n=19.[b)] P=0.0230.

could be obtained by analyzing subgroups based on combined CDK2 and cyclin A or E status. Four subgroups based on positivity or negativity for each antigen were assigned. Kaplan-Meier curves for the overall survival are presented in Fig. 5, D and E. The differences were mostly not statistically significant, but the survival rate of 16 patients with tumors that were negative for both CDK2 expression and cyclin A expression was signifi-

Table III. Contribution of Various Potential Prognostic Factors to Disease-free Survival by Cox Regression Analysis in 103 Oral Cancers

Variable	RR[a)]	95% CI[b)]	*P*
T classification (T3, T4)	1.149	0.553–2.388	0.709
N classification (N+)	2.808	1.357–5.813	0.0054
Tumor differentiation (poorly)	1.557	0.749–3.236	0.2353
Mode of invasion (grade 3/4)	6.329	2.155–18.518	0.0008
CDK2 (≥5%)	2.663	1.013–6.998	0.0469

a) RR, risk ratio.
b) CI, 95%, confidence interval for the relative risk ratio.

cantly higher than that of patients with both CDK2 and cyclin A positivity and patients with either CDK2 or cyclin A positivity (P=0.0392). The survival rate of 20 patients with tumors that were negative for CDK2 expression and positive for cyclin E expression was statistically higher than that of the other patients (P=0.0230). In univariate analysis, stage grouping was significantly associated with disease-free survival (P=0.002 by log-rank test). On the other hand, tumor differentiation (P=0.0114) and tumor invasion (P=0.0001) were also significantly associated with survival. When a Cox proportional hazards model was constructed that included T stage, N stage, tumor differentiation, tumor invasion, and CDK2 expression, CDK2 was an independent predictor of survival (P=0.0469, risk ratio (RR)=2.663) (Table III).

DISCUSSION

It has been suggested that pRB regulates the cell cycle by restricting DNA replication,[31] while CDK2 is a kinase which forms complexes with cyclins[32] and modulates pRB function. Among the higher eukaryotes, CDK2 is considered to be one of the key enzymes that phosphorylate and inactivate pRB at the G1/S transition and allow the cells to enter S phase.[33, 34] The expression of CDK2 and abundance of the cyclin E/CDK2 complex are maximal at the G1/S transition,[8] and cyclin A is expressed in association with CDK2 for progression through the S phase.[13]

The expression of CDK2 in normal epithelia and epithelial dysplasia was absent or generally weak in the basal cell layer (<5% positive cells), but CDK2-staining was dramatically increased in oral SCC. The results of immunohistochemical staining and western blotting data clearly showed that the amount of CDK2, which plays an important role in regulating the S-phase entry, was increased in oral cancer tissues. CDK2 overexpression in oral SCC may elevate pRB phosphorylation and permit more rapid entry of the cancer cells into S phase. The clinicopathological survey of oral SCC in this study showed that the incidence of CDK2 expression was high in the poorly differentiated lesions, and was associated with the mode of tumor invasion, lymph node involvement and survival. This appears to indicate that change in CDK2 expression is related to the progression of oral cancer.

Cyclin A is expressed from the late G1 phase through the M phase of the cell cycle.[32] Cyclin A forms a complex with CDK2 in the G1/S phase and with CDC2 in the M phase.[32, 35] The cyclin A/CDK2 complex inactivates pRB by phosphorylation, facilitating the G1/S transition.[4] The cyclin A/CDC2 complex phosphorylates the nuclear membrane protein laminin, resulting in breakdown of the nuclear membrane.[35] Overexpression of cyclin A has been observed in colorectal carcinoma,[36] breast carcinoma,[37] and uterine carcinoma.[22] Thus, cyclin A is an important positive regulator of the cell cycle. In this study, positive immunohistochemical staining for cyclin A was detected in more than 50% of cases of severe epithelial dysplasia and oral SCC, and the cyclin A expression correlated with the PCNA labeling index. In previous studies on breast[38] lung[20] and uterine[22] cancers, similar correlations between cyclin A expression and cell proliferation have also been found. One possibility is that this positive cyclin A staining simply reflects an increase in the proportion of the cell populations in the S through G2/M phase.

Cyclin E is expressed in the G1 phase of the cell cycle, and forms a complex with CDK2.[32] The kinase activity generated by this complex also phosphorylates pRB, resulting in progression of the G1 phase of the cell cycle.[4] Thus, overexpression of cyclin E/CDK2 is theoretically oncogenic. In our study, only a limited number of cyclin E-positive cells was observed in the normal squamous epithelia, whereas considerable numbers of cells in severe epithelial dysplasia and SCCs overexpressed cyclin E. While overexpression of cyclin E has also been noticed in breast,[39] endometrial,[40] uterine,[22] and lung[20, 41] cancers, the role of this protein is not clear.

Amplification/overexpression of cyclin A, D1 or E in human tumors has been variously described in the literature as a positive or negative prognostic factor.[18–20, 23, 42] In our studies, CDK2 positivity was closely correlated with the mode of tumor invasion, tumor differentiation, and lymph node involvement, and was shown to correlate significantly with shorter survival. No significant correlation was found between only cyclin A or E expression and the overall survival. However, the observed correlation between the clinical outcome and tumors displaying the combination of low expression of both CDK2 and cyclin A is an interesting and potentially important finding in this study. It raises the question as to whether the combination of CDK2 and cyclin A expression is potentially a more accurate prognostic tumor marker than either parameter alone in patients with oral cancers. Our data also showed

that the combination of low expression of CDK2 and positive cyclin E expression correlated to longer survival. Possibly, cyclin E plays a role in cellular differentiation, as described for neuronal or osteoblastic cell line differentiation,[43, 44] and it may eventually be associated with better prognosis.

Collectively, our results showed that the expression of CDK2 could serve as a novel biomarker that can predict the proliferative activity, and ultimately the patients' prognosis in oral carcinoma. Likewise, cyclin A could also be a predictor of proliferative activity, and it seems to appear at an earlier stage than CDK2 in carcinogenesis.

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