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Differential expression of E-cadherin in metastatic lesions comparing to primary oral squamous cell carcinoma

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BACKGROUND: The main cause of treatment failure in resectable oral squamous cell carcinoma (OSCC) is metastasis. E-cadherin (E-cad) plays a principal role in cell adhesion and motility, and is associated with OSCC progression. The aim of this study was to investigate the clinical significance of E-cad expression in OSCC with lymph node metastasis which had radical neck dissection done.

METHOD: Immunohistochemistry was used to detect E-cad expression in normal oral mucosa (NOM) ($n = 10$), oral precancerous lesions (OPLs) ($n = 20$), primary OSCC ($n = 45$), and their paired metastatic lesions ($n = 45$). E-cad immunoreactivity correlated with the clinicopathologic features.

RESULTS: E-cadherin immunoreactivity was progressively reduced in the NOM followed by OPLs and primary OSCC (58%). It decreased significantly in the advanced stages of OSCC. However, the increase in E-cad immunoreactivity was observed in the majority (60%) of metastatic lesions in relation to primary OSCC. Patients with such increased or positive immunoreactivity of E-cad in metastatic lesions exhibited worse prognosis.

CONCLUSION: The findings suggested a dynamic change in E-cad immunoreactivity during tumorigenesis and metastasis of OSCC. In a multivariate analysis, E-cad immunoreactivity in metastasis lesions (odds ratio 3.74, 95% CI 1.15–14.67; $P = 0.040$) implied the potential role of mortality predictors for OSCC cases with nodal involvement.

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the leading cancers worldwide and is becoming a great health threat in Taiwan (1). Although advanced surgical techniques and new anticancer drugs are developed constantly, the overall postoperative survival of OSCC patients did not improve much, with a low 5-year survival rate of 25–30%. The poor prognosis of OSCC patients is highly related to the degree of cancer cell dissemination from the primary site. This highlights the importance of having a more reliable and efficient metastasis marker to monitor the prognosis in addition to the conventional grading systems (1).

Epithelial calcium dependent adhesion molecule (E-cadherin; E-cad), a calcium-dependent transmembrane glycoprotein, plays a crucial role in cell adhesion (2, 3). E-cad maintains normal epithelial polarity, anchoring the cytoskeleton of adjacent cells via cytoplasm protein in catenin superfamilies (4). Loss of cellular adhesion is involved in tumor morphology, invasive phenotypes, and distant metastasis. Aberrant expression of E-cad has been reported in various malignancies (4–8). It is also associated with the advanced stage, lymphatic or hematogeneous spreading (9). Differential expression with cytoplasm shifting of E-cad has been demonstrated in OSCCs and in some oral precancerous lesions (OPLs), compared with the normal oral mucosa (NOM) (10). However, sequential and dynamic expressions of E-cad during distant dissemination have not yet been demonstrated. An *in vitro* study showed that cadherin-mediated intercellular adhesions promoted anchorage-independent growth and suppressed apoptosis in an OSCC cell (11). Whether E-cad expression in tumor cells bestows survival advantages for OSCC cells, when re-colonizing in lymph nodes, has not been addressed *in vivo*. In this study, progressive reduction of E-cad immunoreactivity in consecutive oral carcinogenesis processes from NOM, to OPL and OSCC was defined. In addition, comparisons between primary OSCC (POSCC) and their paired metastatic OSCC (MOSCC) specified that a large fraction of MOSCC showed the reappearance of E-cad immunoreactivity in

lymph nodes. Interestingly, such upregulation would give a poor prognosis prediction.

Material and methods

Samples

All specimens were harvested from the patients treated in Veterans General Hospital-Taipei and Taipei Mackay Memorial Hospital. They were collected between 1999 January and 2005 January. Subjects did not receive any radiation or chemotherapy prior to surgery and following sampling of the lymph node.

All samples were fixed and sectioned following standard protocols (12). Ten NOMs, which were normal controls, and 20 OPLs were used. In addition, 45 pathologically proved late-stage OSCCs, which underwent radical neck dissection treatment and resection of the primary lesion, provided tissue sections for both primary and paired metastatic lesions in this research. The clinicopathologic features, including age, site, areca use, tumor differentiation, tumor size, lymph node metastasis and survival data, of the OSCC subjects were recorded.

Immunohistochemistry (IHC)

Sections were deparaffinized in xylene and re-hydrated by immersion in a graded series of ethanol dilutions. All slides were immersed in 10 mM sodium citrate solution, in a microwave oven, to retrieve antigenicity. Sections were quenched with 3% fresh H_2O_2 for 10 min to inhibit endogenous tissue peroxidase activity, and rinsed with 1x phosphate-buffered saline (PBS) for 5 min twice. Sections were further incubated in blocking serum for 30 min and then for 2.5 h with anti-E-cad antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) with a 1:400 dilution in a humid chamber. After washing with 1x PBS for 5 min twice, sections were then incubated with biotinylated secondary antibody solution for 30 min. LSAB2® streptavidin-peroxidase detection reagent (DAKO, Santa Barbara, CA, USA) was subsequently added evenly over the sections and incubated for 30 min. The sections were washed with 1x PBS for 5 min twice, incubated with freshly prepared amino ethyl carbazole (Zymed, South San Francisco, CA, USA) substrate solution for 15 min and then washed with 1x PBS. All IHC staining was performed at room temperature. The sections were finally counterstained with hematoxylin, washed with 1x PBS and deionized distilled water, and then mounted. As an internal positive control for E-cad staining, membranous staining of normal squamous epithelium harvested from healthy adult patient was used. As a negative control for stains, normal mouse immunoglobulin of the same class was used instead of the primary antibody. Meanwhile, the most representative area of specimen section such as the central part of the tumor nest was chosen for E-cad immunoreactivity grading. The tissues of paired primary tumors and lymph node were stained immunohistochemically together with the same reagents by simultaneous procedures. Although not all sections were stained at the same time, most tissues, especially those with undetermined results, were stained twice for reproducible expression level. The percentage of immunoreactive cells, with either membranous or cytosolic E-cad, was estimated in five random 400 microscopic fields (12). The tissue sections were categorized as E-cad (++, 2), E-cad (+, 1) or E-cad (−, 0), when over 50%, 10–50%, and less than 10% of the tumor cells were positive for immunostaining, respectively. For reaching a more precise judgment of positive expression, two independent observers blinded to the stage and patient profiles reviewed the immunohistochemically stained sections.

Statistical analysis

The significance of E-cad immunoreactivity in different groups or with different variables was investigated using Fisher's exact test. The survival status was analyzed with Kaplan–Meier analysis. $P < 0.05$ was considered to be statistically significant.

Results

Characteristics of cases

The age of OSCC cases ranged from 39 to 71 years with an average of 54 ± 7. A marked gender difference between males and females (41 vs. 4) was consistent with a previous report on OSCC in Taiwan (13). The tissue samples were harvested from the buccal mucosa (19 cases), tongue (11 cases), gingiva (5 cases), mouth floor (9 cases), and palate (1 case). Stage IV tumors were presented in 33 (73%) cases and stage III tumors in 12 cases (27%). Seventeen cases (38%) had an extensive T4 tumor mass. Twenty-eight (62%) cases had the tumor size ranging from T1 to T3.

The histopathologic grading showed that 12 (27%) OSCCs were well differentiated, 17 (38%) were moderately differentiated, and 16 (36%) were poorly differentiated (Table 1). Twenty (44%) OSCC cases died at the end of the observation period. The histopathologic diagnosis of OPL was the presence of epithelial hyperplasia and/or hyperkeratosis in 11 cases and epithelial dysplasia in nine cases.

E-cad immunoreactivity

E-cadherin immunoreactivity was observed in the membranous or cytosolic compartments of NOM and in the

Table 1 E-cad immunoreactivity in various oral tissues

	E-cad (−)	*E-cad (+) or E-cad (++)*	*P-value**
NOM ($n = 10$)	0	10	
			0.140
OPL ($n = 20$)	5	15	
			0.020
POSCC ($n = 45$)	26	19	
			0.003
MOSCC ($n = 45$)	11	34	

E-cad, E-cadherin; NOM, normal oral mucosa; OPL, oral precancerous lesion; POSCC, primary oral squamous cell carcinoma; MOSCC, metastatic oral squamous cell carcinoma.
*Fisher's exact test.

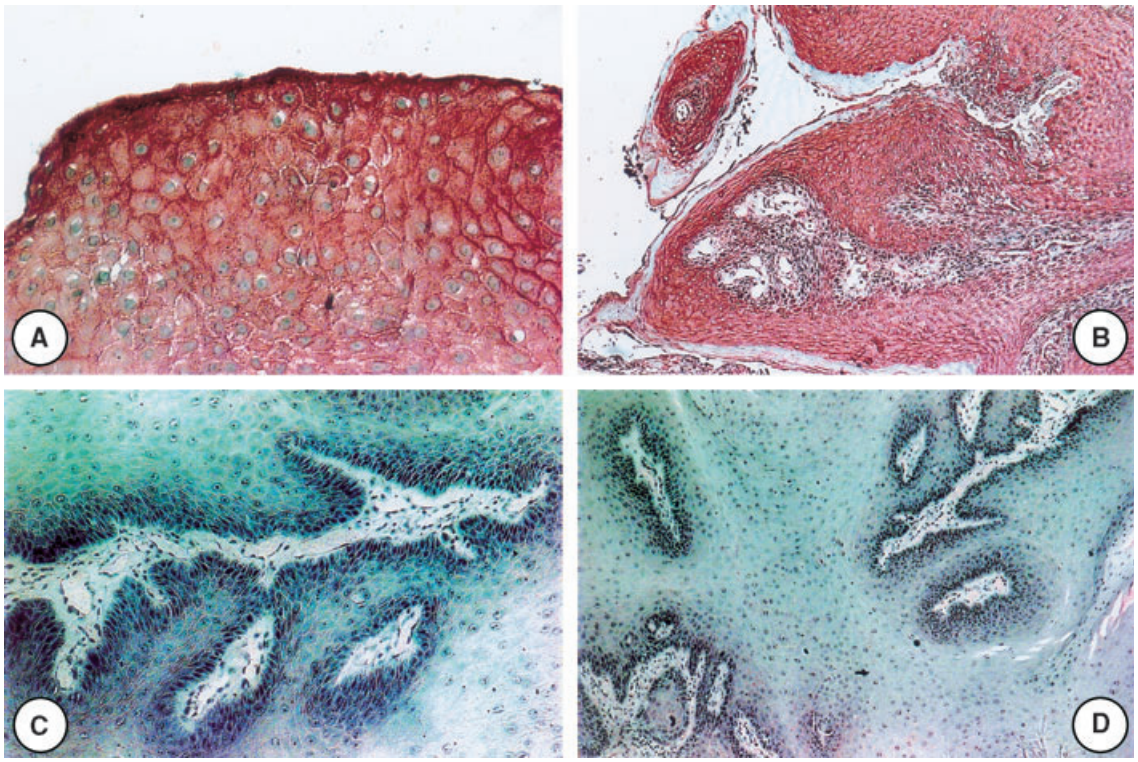

Figure 1 E-cadherin (E-cad) immunoreactivity in normal oral mucosa (NOM; A) and oral precancerous lesions (OPLs; B–D). (A) A NOM shows extensive membranous and/or cytosolic E-cad immunoreactivity in nearly full thickness of epithelium, scoring E-cad (++). (B) A verrucous OPL diagnosed as epithelial hyperplasia and hyperkeratosis exhibits E-cad (++). (C, D) OPLs diagnosed as mild epithelial dysplasia and epithelial hyperplasia, respectively, exhibit E-cad (−). (A and C, 200×; B and D, 100×).

majority of OPL. E-cad immunoreactivity was scored according to the number of cells exhibiting immunostaining. All NOM exhibited E-cad (++) (Fig. 1A). E-cad (+) or E-cad (++) appeared in 15 (75%) OPL cases, including nine epithelial hyperplasia and hyperkeratosis (Fig. 1B) and six epithelial dysplasia. In contrast, three epithelial dysplasia (Fig. 1C) and two epithelial hyperplasia (Fig. 1D) exhibited E-cad (−). Nineteen (42%) POSCC were E-cad (+) or E-cad (++). The remaining POSCC cases 26 (58%) showed scattered E-cad immunoreactivity and were scored as E-cad (−) (Fig. 2B). Thirty-four (76%) MOSCC were E-cad (+) or E-cad (++) (Fig. 2C,D), whereas the remaining 11 (24%) cases were E-cad (−). E-cad immunoreactivity decreased progressively from NOM (100%) to OPL (75%) to POSCC (42%) (Fig. 3). However, the reducing trend seemed to be reversed in the metastasizing stage (Fig. 3). Significant differences in E-cad immunoreactivity were noted between OPL and POSCC, and between POSCC and MOSCC ($P = 0.02$ and 0.003, respectively, Fisher's exact test, Table 1).

Correlation between E-cad immunoreactivity and clinicopathologic features of POSCC

In POSCC, eight (18%) cases were E-cad (++), 11 (24%) cases were E-cad (+) and 26 (58%) cases were E-cad (−) (Table 2). E-cad (−) was observed in 13 out of 16 (81%) poorly differentiated POSCC, 9 out of 17 (53%) moderately differentiated POSCC and 4 out of 12 (33%) well-differentiated POSCC. Statistically, E-cad (−) in moderately or poorly differentiated tumors was significantly higher than in well-differentiated tumors ($P = 0.027$, Table 2). Similarly, E-cad (−) was statistically significantly higher in most invasive POSCC ($P = 0.014$), POSCC with multiple nodal spreading ($P = 0.006$), and stage IV POSCC ($P = 0.014$, Table 2). E-cad immunoreactivity was not associated with age, gender or site of tumor, and areca use of the cases.

Correlation between E-cad immunoreactivity and survival of OSCC cases

By the end of the follow-up, with a median survivability of 875 days, 20 OSCC cases died of the disease and 25 survived. Twenty-seven (60%) subjects had an increase in E-cad immunoreactivity from POSCC to MOSCC (Table 3). These cases had statistically significantly lower survival rate than the remaining groups (Table 3, $P = 0.003$). Although E-cad immunoreactivity in POSCC is associated with more extensive nodal involvement and the advanced clinical stage, E-cad immunoreactivity did not affect the survivability of POSCC cases (Fig. 4A). However, cases exhibiting E-cad (+) or E-cad (++) in MOSCC had a worse survivability than other groups (Fig. 4B, $P = 0.040$). Besides, patients with E-cad (−) in MOSCC had an odds ratio of 3.74 for better prognosis (95% CI 1.51–14.67; $P = 0.040$). Interestingly, MOSCC cases with an

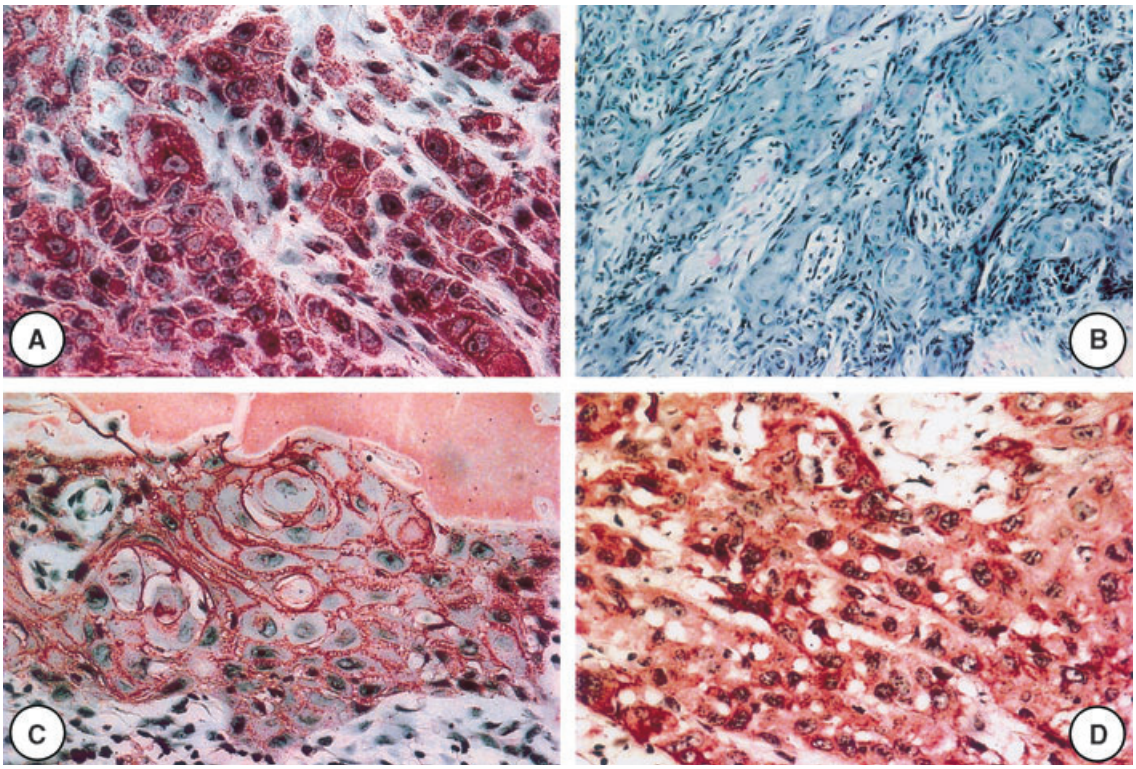

Figure 2 E-cadherin (E-cad) immunoreactivity in oral squamous cell carcinoma (OSCC; A, B) and primary OSCC (POSCC) (C, D, MOSCC). (A) and (C) were obtained from the same patient. (B) and (D) were obtained from another patient. Note that (A, C and D) were E-cad (+ +), while (B) was E-cad (−) (A, C and D, 200×; B, 100×).

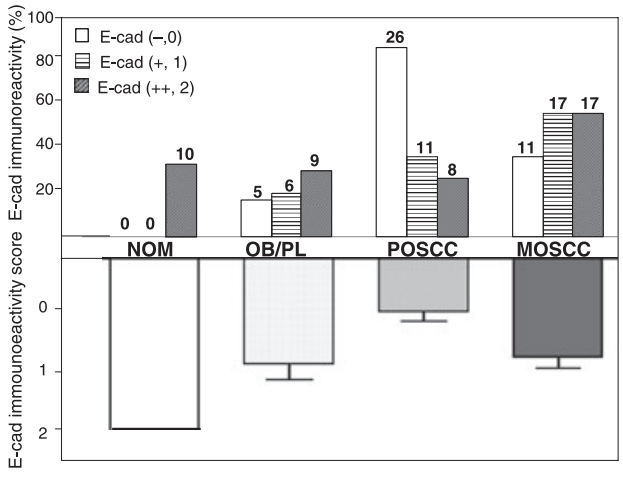

Figure 3 E-cadherin (E-cad) immunoreactivity during oral carcinogenesis and metastasis (upper panel). Note the progressive decrease in E-cad immunoreactivity from normal oral mucosa (NOM) to primary oral squamous cell carcinoma (POSCC), and an increase in metastatic oral squamous cell carcinoma (MOSCC). The numbers in columns are case numbers (lower panel). The immunoreactive scoring of E-cad in each tissue group representing as mean ± standard error.

increased E-cad immunoreactivity had worse survivability in comparison with other groups (Fig. 4C, $P = 0.030$–0.005). In comparison with all other groups, patients with increase in E-cad immunoreactivity in MOSCC had an odds ratio of 6.30 for worse prognosis (95% CI = 1.55–25.65; $P = 0.01$).

Discussion

E-cadherin plays a central role in proliferation, migration, polarization, and differentiation through cell adhesion and signaling regulation in epithelial cells (10, 14). The role of E-cad expression associating with phenotypes and behaviors of OSCC seems to be an important issue for investigation (15). E-cad expression can be regarded as a differentiation marker and has a potential use in grading carcinomas (10, 14, 16). In this study, the absence of E-cad immunoreactivity in primary lesions was observed in 73% stage IV OSCC, which was very different from the 27% in stage III OSCC and 25% in OPL. Moreover, T4, $N \geqq 2$ and poorly differentiated OSCCs had low E-cad immunoreactivity in relation to other groups. Thus, the results substantiated that the downregulation of E-cad is associated with aggressiveness and invasiveness of OSCC (17).

Metastatic process of the malignant tumors involved a complex series of events (3). At the invasion stage, loss of E-cad, integrin and other adhesion molecules seems to be critical for the detachment of tumor cells from the primary lesion (18). However, the re-colonization of metastatic cells in lymph nodes might require an adaptation to environmental challenges such as hypoxia, acidosis, demands for angiogenesis, and the reduction in anchorage (19). Our study demonstrated that the vast majority of metastatic deposits had higher E-cad expression than their counterparts in primary lesions.

Table 2 E-cad immunoreactivity and clinicopathological features in POSCC

	E-cad (−)	*E-cad (+)*	*E-cad (++)*	*P-value**
Age				
≧ 54 ($n = 31$)	16	7	8	0.330
< 54 ($n = 14$)	10	4	0	
Site				
Buccal mucosa ($n = 19$)	10	7	2	0.761
Non-buccal mucosa ($n = 26$)	16	4	6	
Areca use				
User ($n = 42$)	24	10	8	1.000
Non-user ($n = 3$)	2	1	0	
Differentiation[a]				
Well ($n = 12$)	4	5	3	0.027
Moderate ($n = 17$)	9	4	4	
Poor ($n = 16$)	13	2	1	
Size				
T1–T3 ($n = 28$)	12	9	7	0.013
T4 ($n = 17$)	14	2	1	
Lymph node metastasis				
$N = 1$ ($n = 13$)	3	6	4	0.006
$N \geqq 2$ ($n = 32$)	23	5	4	
Stage				
III ($n = 12$)	2		4	0.014
IV ($n = 33$)	24		4	

E-cad, E-cadherin; POSCC, primary oral squamous cell carcinoma.
[a]Moderately and poorly differentiated tumors were regarded as one group for comparison with well-differentiated tumors.
*E-cad (+) and E-cad (++) were regarded as one group to compare with the E-cad (−) cases, Fisher's exact test.

Table 3 E-cad immunoreactivity and prognosis

POSCC to MOSCC	*Survival*	*Death*	*Median survival (days)*	*P-value**
Increased ($n = 27$)	10	17	763	
− to + ($n = 12$)	5	7		
− to ++ ($n = 9$)	3	6		0.003
+ to ++ ($n = 6$)	2	4		
Equal or decreased ($n = 10$)	15	3	989	
− to − ($n = 5$)	4	1		
+ to + ($n = 3$)	3	0		
++ to ++ ($n = 2$)	1	1		
++ to + ($n = 2$)	1	1		
++ to − ($n = 4$)	4	0		
+ to − ($n = 2$)	2	0		

POSCC, primary oral squamous cell carcinoma; MOSCC, metastatic oral squamous cell carcinoma.
*Comparison between increased group and equal or decreased group, Fisher's exact test.

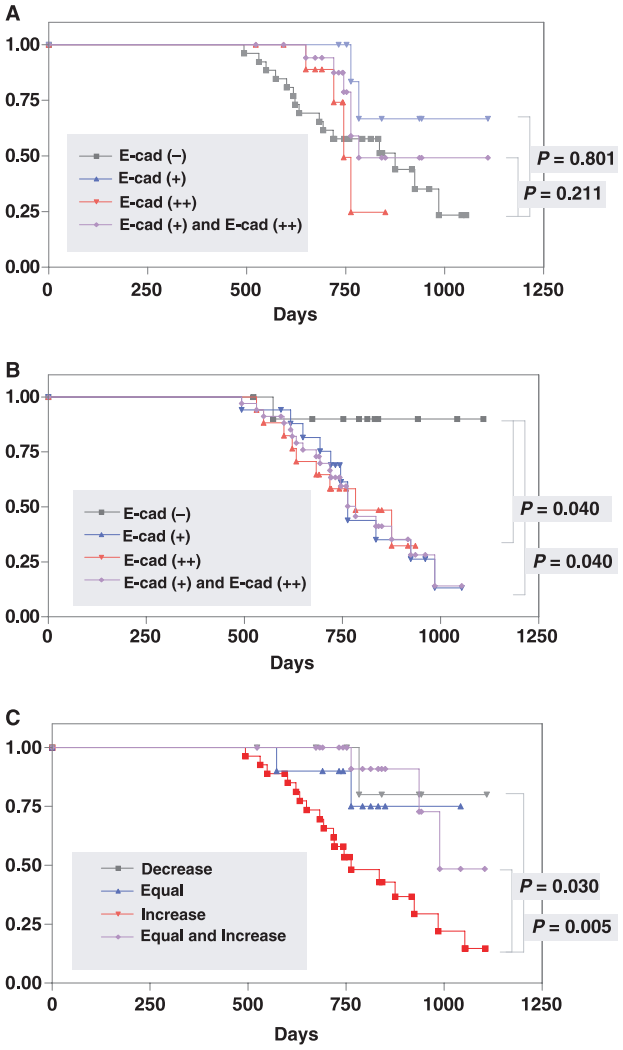

Figure 4 Survival of oral squamous cell carcinoma (OSCC) cases. (A) E-cadherin (E-cad) immunoreactivity in primary oral squamous cell carcinoma (POSCC) vs. survival of cases. No association between various groups was identified. (B) E-cad immunoreactivity in metastatic oral squamous cell carcinoma (MOSCC) was related to survival of cases. MOSCC cases exhibiting E-cad (+) or E-cad (++) had a statistically significant lower survivability than MOSCC cases exhibiting E-cad (−). (C) E-cad immunoreactivity in MOSCC comparing to POSCC in relation to the survival of cases. MOSCC cases exhibiting an increase in E-cad immunoreactivity comparing with POSCC, which had a statistically significant lower survivability than those exhibiting equal or decreased in E-cad immunoreactivity.

The findings agree with a previous finding in breast cancer, denoting that E-cad immunoreactivity was significantly increased in the metastatic lesions compared with the respective primary site (2, 20). It also generally agrees with the upregulation of cadherin complex proteins in carcinoma cell effusions of ovarian cancers when compared with primary tumors (4). Kantak and Kramer (11) have proposed *in vitro* clues that cadherin can help the growth of an OSCC cell in an anchorage-independent system. As the increase in E-cad immunoreactivity is highly associated with a worse prognosis of OSCC, it is likely that the re-expression of E-cad in the lymph node confers advantages to OSCC in metastatic environments.

Promoter hypermethylations bestowing transcription silencing (21, 22), destabilization of catenin binding (23), genomic alterations, such as mutation or deletion, attenuation of membranous protein through Rac activation (24) or other mechanisms (12) have been the major causes underlying E-cad downregulation in epithelial malignancies. Our findings regarding the decrease in E-cad immunoreactivity, occurring mainly during the progression of primary tumors, supported that loss of

E-cad allows neoplastic cells to invade (25). The decision/critical step of successful growth at metastasis sites was presumed to be E-cad re-expression, which facilitates intercellular adhesion and colonization. As genetic alterations cause irreversible changes, regulation from promoter methylation, catenins, Rac or other elements could be the only reversible change in E-cad immunoreactivity.

Lymph node metastasis is one of the most important parameters in defining the outcomes of OSCC. In this cohort study, OSCC with nodal involvement further decreased the survivability. We found that the increase of E-cad immunoreactivity in metastatic lesions would predict the worst prognosis in this subset. Preliminary findings imply that the dynamic E-cad changes could have the potential to improve the outcome of OSCC therapy. As the mechanism of E-cad regulation in metastatic lesions has become clearer and better understood, anti-E-cad regimens may support surgical resections in improving patient survival.

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