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The molecular markers for prognostic evaluation of areca-associated buccal squamous cell carcinoma

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BACKGROUND: Buccal squamous cell carcinoma (BSCC) is the most frequently occurring oral cancer in Asians due to the popularity of areca use in this area. The aim of the present study was to evaluate the survival of areca-associated BSCC associated with multiple molecular markers.

METHODS: Using immunohistochemistry, we evaluated the survival of a cohort of 55 patients with BSCC being followed long term, as correlated to the expression of variable markers.

RESULTS: We found that p53, p21, Rb, cyclin D1 (CCD1), MDM2, and γ-catenin were positive in 81, 60, 70, 31, 88, and 44% of patients, respectively. Subjects with −ve immunoreactivity for CCD1, and +ve immunoreactivity for MDM2 and γ-catenin had significantly better survival than subjects with the opposite immunoreactive pattern. Kaplan–Meier survival curves confirmed this association.

CONCLUSION: The data indicate that expression of CCD1, MDM2, and γ-catenin might serve as potential prognostic markers for BSCC in areca-using patients.

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Keywords: areca; buccal mucosa; catenin; cyclin D1; MDM2, mouth neoplasm; squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is a very frequent cancer in most Asian countries, including Taiwan, due to the popularity of areca use (1–3). In areca users, buccal squamous cell carcinoma (BSCC) is the most common subtype of OSCC, accounting for more than 60% of cases (4). OSCC is believed to develop through sequential and/or cumulative gene alterations (5, 6). The interactions between activation of oncogenes and loss of function of tumor suppressor genes appear to drive uncontrolled cell growth and invasion (7, 8). These genetic events may also serve as diagnostic or prognostic markers.

The protein p53, the key tumor suppressor regulating cell cycle arrest and apoptosis, is altered in more than 50% of human malignancies. Immunoreactivity for an abnormal p53 protein is prevalent in OSCC, usually related to a functional abrogation through mutation, phosphorylation or binding to oncogenic proteins (3, 9, 10). p21 protein, also called Waf1 or Cip1, is a cyclin-dependent kinase inhibitor which functions as the main downstream effector of p53 protein. Immunoreactivity for p21 has been observed in a variety of human cancers (11, 12). We have identified aberrant p53/p21 immunoreactivity as an adverse prognostic predictor in the outcome of oral pre-cancerous lesions (13). A human homologue of the mouse double minute-2 (MDM2) protein, an oncogenic protein binding to p53, serves as a molecular chaperon that degrades p53 proteins. There is controversy over variation in the incidence of MDM2 expression in OSCC, and other head and neck squamous cell carcinomas (HNSCC) (14–17). Overexpression of MDM2, representing an alternative mechanism for p53 inactivation, has been identified in a high percentage (69–78%) of OSCC (14, 16). However, Millon et al. (17) identified the frequent (90%) loss of MDM2 expression in HNSCC.

Another key axis for cell cycle regulation at the G_1/S transition stage is the retinoblastoma protein (Rb) pathway. The binding of cyclin D to cdk 4 and cdk 6 regulates Rb phosphorylation, which in turn drives the release of E2F and subsequent cell cycle progression through the G_1/S checkpoint (18–20). Altered Rb expression has been observed in OSCC, although its implication in disease progression has hitherto been undefined (12, 15, 21, 22). The *cyclin D1* (*CCD1*) gene, mapped to 11q13, is profoundly amplified and overexpressed in a variety of cancers, including OSCC (23). In most studies, its expression has been viewed as an unfavorable survival factor. However, CCD1 is also involved in the control of apoptosis and growth suppression (24). It is obvious that the multifunctional properties of CCD1 may contribute to different effects on cell proliferation and clinical outcome. Lam et al.

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(25) have demonstrated a site difference of CCD1 overexpression in OSCC. Carcinomas of the palate, floor of the mouth, and gingiva tend to have a higher incidence of CCD1 overexpression than tumors at other sites. It is of importance to clarify the expression of CCD1 in BSCC and understand the prognostic significance.

Cell–cell adhesion is mediated by cadherins, which form a complex with catenins and cytoskeletons. Down regulation of cadherins has frequently been detected in many human carcinomas, in which it has been associated with tumor progression and invasion. It has also been shown that loss of β- and γ-catenin function is associated with tumor metastasis (26). Increased accumulation of β-catenin caused by mutation of the APC gene or alteration of the consensus motif for GSK-3β phosphorylation as a result of the β-catenin mutation itself plays pivotal roles in carcinogenesis (27). Therefore, it has also been suggested that β-catenin functions as a regulator of signal transduction in addition to its classical role as a regulator of the cell adhesion system (27). γ-Catenin is also associated with the APC and exerts signaling activity similar to that of β-catenin in animal model. In human tumors, in contrast to β-catenin, whether γ-catenin is associated with signaling or functions as an element of cell attachment is still unclear. Given the importance of cell cycle regulation and cell adhesion in OSCC formation, we measured the immunoreactivity of p53, p21, Rb, MDM2, CCD1, and γ-catenin proteins in areca-associated BSCC in order to determine their prognostic significance.

Materials and methods

Samples

A total of 55 pathologically diagnosed BSCC tissue samples were obtained from patients at the Department of Oral and Maxillofacial Surgery of the Taichung Veterans General Hospital from 1985 to 1996. All patients were areca chewers. None had received any treatment for OSCC before surgical intervention. The pre-operative evaluation included age, tumor size, regional lymph node metastasis (LNM) and clinical stage. Postoperatively, adjuvant radiation therapy and/or chemotherapy were employed in some patients with advanced stage disease. The patients were followed periodically.

Immunohistochemistry (IHC)

A total of 12 serial 5 μm-thick paraffin sections containing at least 1 cm^2 of tumor tissue were prepared from a most representative BSCC tissue block per subject. Following dewaxing and rehydrating, endogenous peroxidase activity was inactivated with 1% H_2O_2. Antigenicity was retrieved by microwave heating in 10 mM sodium citrate solution for 3 min. After blocking nonspecific binding with 2% dry milk in PBS, the first six serial sections were incubated with the following monoclonal antibodies in the following order: anti-p53 (clone DO-7, Dako, Glostrup, Denmark) at 1:150 dilution; anti-p21 (clone EA10, Calbiochem, San Diego, CA, USA) at 1:50 dilution; anti-Rb (clone G3-245, Pharmingen, San Diego, CA, USA) at 1:150 dilution; anti-MDM2 (Novocastra Laboratory, Newcastle upon Tyne, UK) at 1:250 dilution; anti-CCD1 (clone p2D11F11, Novocastra Laboratory, Newcastle upon Tyne, UK) at 1:100 dilution; and anti-γ-catenin (Transduction Laboratory, San Diego, CA, USA) at 1:200 dilution. This was followed by biotinylated secondary antibody and avidin–biotin complex conjugated to horseradish peroxidase (Vectastain Elite ABC kit, Vector, Burlingame, CA, USA). Sections were then treated with 1 mg/ml chromogen AEC (Zymed, South San Francisco, CA, USA) and 0.01% H_2O_2 for 90–180 s. Sections were lightly counterstained with hematoxylin. Random identification of tumor nests was achieved by delineating numerous small circles on the glass slides prior to microscopic examination. Small nests of up to 100 cells were identified at random and the total number of cells was counted along with the number of cells exhibiting distinctive nucleic, cytoplasmic, or membrane immunoreactivity. A total of 10 random high-power (×400) fields were examined on each slide. Specimens containing <10% positive cells were defined as −ve (negative) for expression. Specimens containing 10–50%, and ≥50% positive cells were defined as + (weakly positive) and ++ (strongly positive) for immunoreactivity, respectively. For our analysis, cases with + or ++ immunoreactivity were classified as +ve (positive) for expression. Xenographic tumor tissue derived from the OC2 OSCC cell line carrying a p53 mutation served as a positive control for p53, MDM2, and CCD1 immunoreactivity. Positive controls for p21, Rb, and γ-catenin were normal gingival epithelium. Negative controls were obtained by omitting primary antibodies. Immunohistochemistry and scoring were repeatedly performed on all cases exhibiting −ve expression for each marker and cases exhibiting +ve expression for all markers using the second set of serial sections.

Statistical analysis

For the categorical variants, the *t*-test, χ^2-analysis or Fischer's exact test were used. Cumulative survival was analyzed with the Kaplan–Meier product-limit method. The duration of survival was measured from the beginning of treatment to the time of death or the last follow-up. Outcome analysis was based on the relative risk regression model of Cox. A $P < 0.05$ was considered to be statistically significant.

Results

The age of the patients at diagnosis ranged from 31 to 64 years, with an average of 49.6 years. Thirty-two patients presented with advanced (stages III and IV) tumors and 23 had early tumors. During the follow-up period ranging from 4 to 147 months, 26 patients remained tumor-free and 29 patients died mostly of recurrent cancer. Table 1 summarizes the clinicopathological parameters according to outcome. LNM was identified as a prognostic determinant of BSCC, since 62% of subjects with LNM died during the follow-up

Table 1 Clinicopathological parameters

	Survived (n = 26)	*Died (n = 29)*	*P**
Age (year) (mean ± SE)	32–61 (49.6 ± 9.0)	32–64 (50.3 ± 9.0)	ns
Follow-up (months) (mean ± SE)	92.6 ± 11.5	18.6 ± 7.4	
T status			
1–2 ($n = 45$)	47% ($n = 21$)	53% ($n = 24$)	ns
3–4 ($n = 10$)	50% ($n = 5$)	50% ($n = 5$)	ns
LNM			
$N = 0$ ($n = 28$)	65% ($n = 17$)	38% ($n = 11$)	<0.001
$N > 0$ ($n = 27$)	35% ($n = 9$)	62% ($n = 18$)	<0.001
Stage			
I–II ($n = 23$)	56% ($n = 13$)	44% ($n = 10$)	ns
III–IV ($n = 32$)	41% ($n = 13$)	59% ($n = 19$)	ns

*Fisher's exact test; LNM, lymph node metastasis; ns, not significant.

period (Table 1, $P < 0.001$). Kaplan–Meier curves also demonstrated a significantly worse prognosis with regional LNM (Fig. 2A, $P < 0.001$). No significant variation in outcome was detected in association with age, tumor size, or clinical stage.

Immunohistochemistry (IHC) identified nuclear staining for p53, p21, Rb, CCD1, MDM2, and membrane staining for γ-catenin (Fig. 1). Weak cytoplasmic immunoreactivity of p53, p21, Rb, and CCD1 in addition to nuclear staining was occasionally seen (Fig. 1A–D). The cytoplasmic immunoreactivity of MDM2 was intensive, whereas in 60% of cases MDM2 immunoreactivity was scored as '+' (Fig. 1E; Table 2). In addition to membranous immunoreactivity, focal areas also exhibited cytoplasmic immunoreactivity of γ-catenin (Fig. 1F). The cells exhibiting cytoplasmic immunoreactivity but no nuclear or membranous immunoreactivity were counted as −ve. The +ve expression rates for p53, p21, Rb, CCD1, MDM2, and γ-catenin in BSCC were 81, 60, 70, 31, 88, and 44%, respectively (Table 2). For five cases exhibiting +ve expression for all markers in the first experiment, the counterpart IHC in the second set was scored to evaluate the concordance. There was an 87% (26/30) concordance in the immunoreactivity and complete consensus in expression between the two independent experiment suggesting the reproducibility. No case being originally evaluated as +ve has been re-scored as −ve in the second experiment. A complete consensus between the two experiment sets was also obtained in cases exhibiting −ve expression. The survived subjects had a significantly higher rate in CCD1 −ve expression than those died subjects (Table 2, $P < 0.001$). Likewise, the survived subjects had a significantly higher rate in MDM2 +ve or γ-catenin +ve expression than those died subjects (Table 2, $P = 0.01$ and <0.01, respectively). Kaplan–Meier analysis also revealed that subjects with CCD1-negative, MDM2-positive, or γ-catenin-positive expression had better survival ratio (Fig. 2B–D, $P = 0.001$, 0.01, and <0.01, respectively). Differences in the expression of p53, p21, and Rb did not correlate with differences in survival.

Table 2 Immunohistochemistry

		Expression (%)		*Immunoreactivity (%)*		
		+ve	*−ve*	*(+)*	*(++)*	*P**
p53	Total	81	19	23	58	ns
	Survived	80	20	24	56	
	Died	82	18	21	61	
p21	Total	60	40	38	22	ns
	Survived	64	36	52	12	
	Died	54	46	25	29	
Rb	Total	70	30	46	24	ns
	Survived	72	28	56	16	
	Died	68	32	36	32	
CCD1	Total	31	69	27	4	<0.001
	Survived	8	92	8	0	
	Died	52	48	45	7	
MDM2	Total	88	12	60	28	0.01
	Survived	100	0	76	24	
	Died	78	22	44	34	
γ-Catenin	Total	44	56	44	0	<0.01
	Survived	54	36	54	0	
	Died	24	76	24	0	

*Analysis performed on −ve and +ve, Fisher's exact test.
ns, not significant.

To assess the CCD1, MDM2, and γ-catenin markers in prognostic evaluation, combined analysis was performed (Fig. 2E–H). Figure 2(F,H) illustrates that the combined use of CCD1/MDM2 and CCD1/MDM2/γ-catenin expression enhanced the prediction of survival as compared to use of each individual marker alone. Subjects who were CCD −ve/MDM2 +ve/γ-catenin +ve demonstrated 100% 10-year survival, while those with the opposite pattern had all died by 4 years (Fig. 2H, $P < 0.0001$). The combined analysis of CCD1/MDM2 appeared invalid due to the limited sample size of the CCD1 +ve/MDM2 −ve group (Fig. 2E).

A univariant regression analysis based on the Cox proportional model was carried out to access the prognostic value of the clinicopathological parameters and the expression of markers for the relationship to survival. In this analysis, LNM together with CCD1 +ve, MDM2 −ve, and γ-catenin −ve in expression and their combinational situation were confirmed to be associated with increased risk (Table 3).

We further correlated the expression of markers with clinicopathological parameters at diagnosis including age, size of the primary lesion, LNM, and stage. A significant correlation was identified between the expression of Rb and γ-catenin with smaller tumor size and earlier clinical stage when compared with cases lacking those two markers ($P < 0.05$, Table 4). A gradual loss of expression of both genes was observed with larger primary lesions and later clinical stages. No marker was significantly associated with LNM, nor was there a significant correlation between the expression of the other genes and patients' clinicopathological parameters. There was a correlation between p53 and p21 expression ($P = 0.01$), but not between p53 and MDM2 expression, or between Rb and CCD1 expression (detailed analysis not shown).

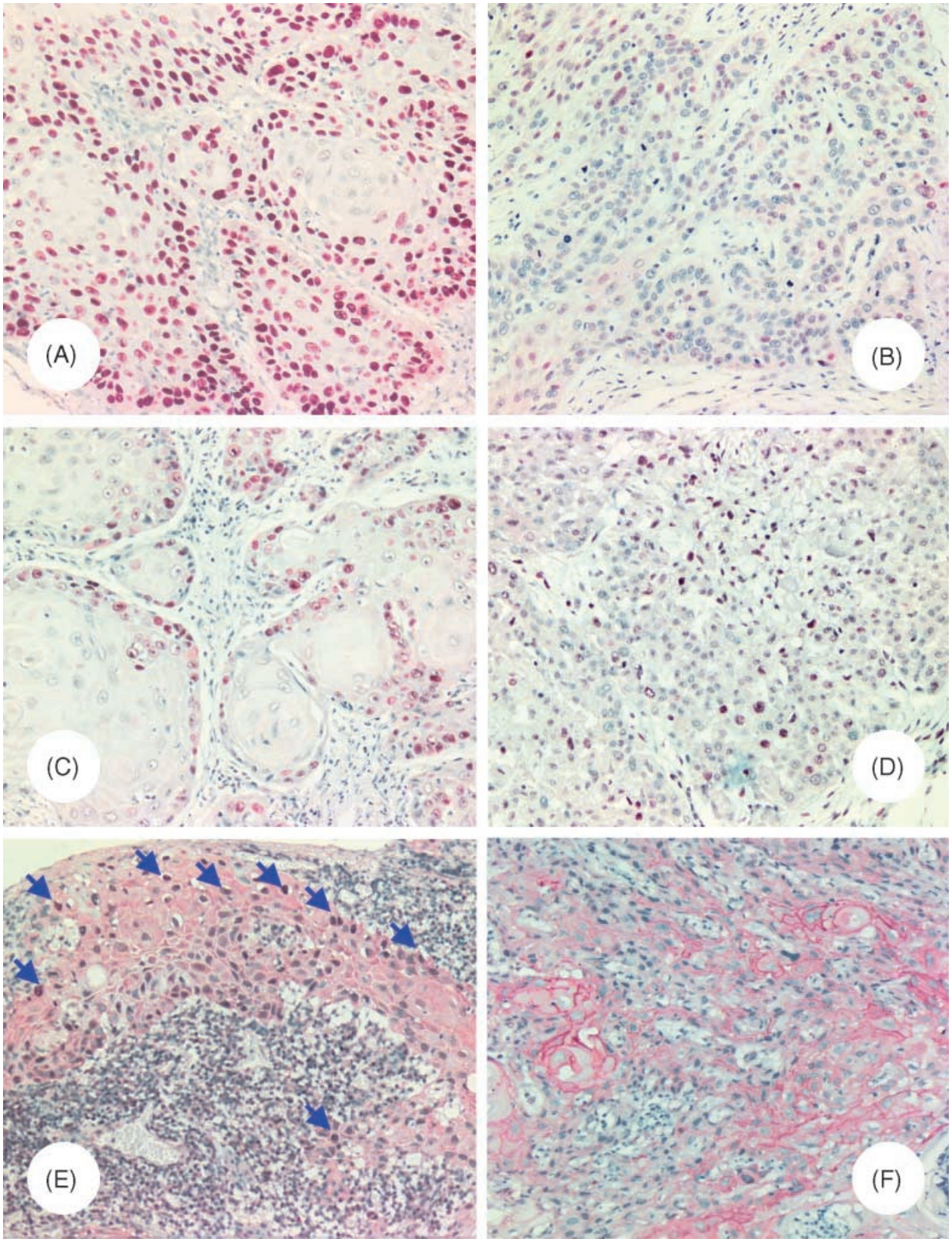

Figure 1 IHC. Positive immunoreactivity of p53, p21, Rb, CCD1, MDM2 and γ-catenin depicted in (A)–(E), respectively. (A) and (C); (B) and (D); and (E) and (F) are each obtained from the same individual. Note the remarkable cytoplasmic immunoreactivity of MDM2 and γ-catenin. Arrows in (E) depict representative MDM2 nuclear immunoreactivity. (A–F, $\times$200).

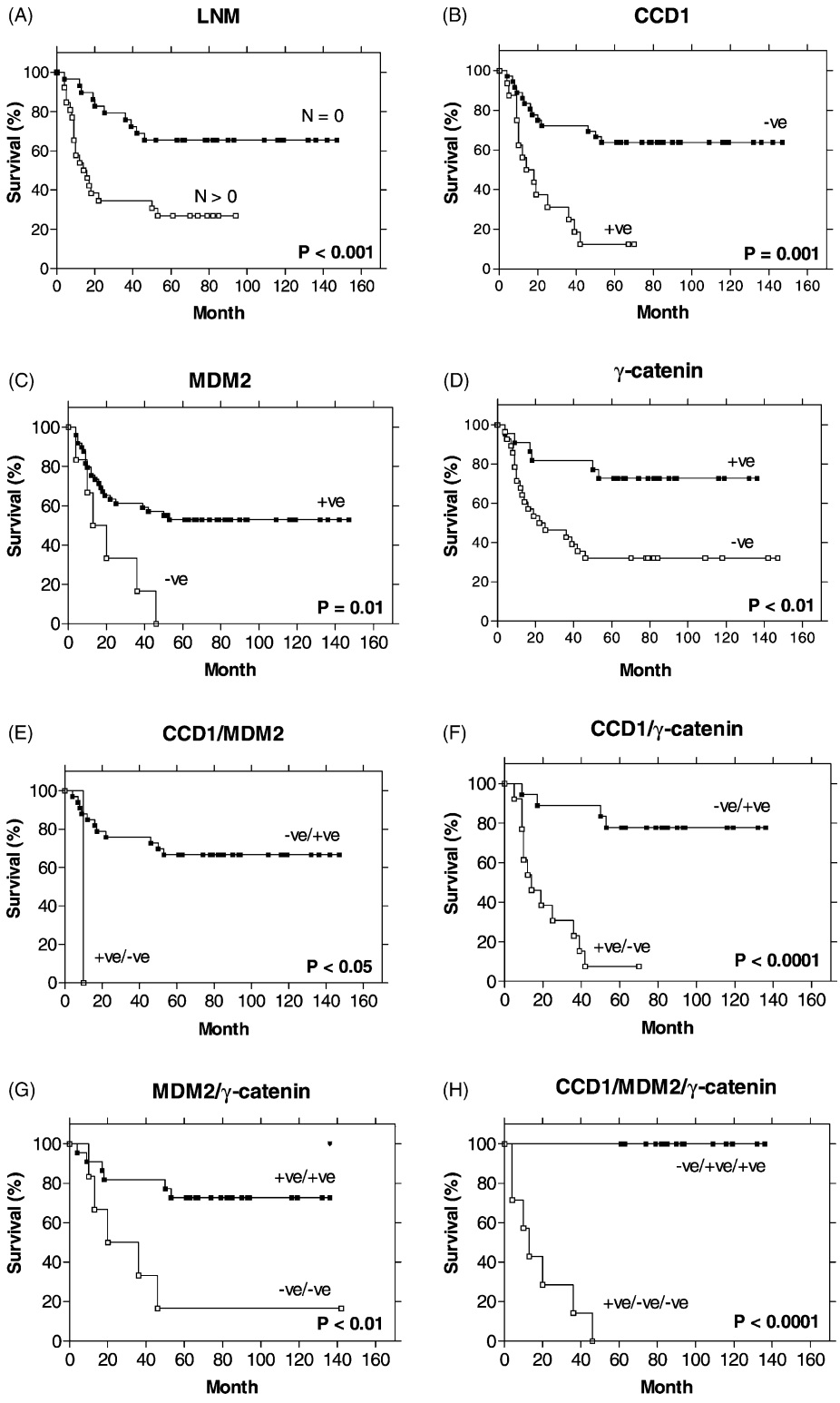

Figure 2 Kaplan–Meier survival analysis using (A) LNM, and expression of (B) CCD1, (C) MDM2, and (D) γ-catenin; and Kaplan–Meier survival analysis using combined expression status of (E) CCD1/MDM2, (F) CCD1/γ-catenin, (G) MDM2/γ-catenin, and (H) CCD1/MDM2/γ-catenin.

Discussion

Areca use is a unique geographically linked habit that is popular in Asia. Areca-associated OSCC appears to differ from that in industrialized countries where the principal etiologic factors are tobacco use and alcohol consumption. For example, mutation and overexpression of the *ras* oncogene have frequently been observed

Table 3 Coxanalysis of potential prognostic factors of BSCC survival

	n	*Relative risk (95% confidence intervals)*	*P*
Age (≧50 vs. <50)	55	0.939 (0.449–1.96)	ns
T status (3–4 vs. 1–2)	55	1.10 (0.654–2.53)	ns
LNM ($N > 0$ vs. $N = 0$)	55	3.35 (1.72–8.04)	<0.001
Stage (III–IV vs. I–II)	55	1.32 (0.87–2.85)	ns
p53 (+ve vs. −ve)	55	0.776 (0.366–1.62)	ns
p21 (+ve vs. −ve)	55	0.843 (0.512–1.94)	ns
Rb (+ve vs. −ve)	55	0.949 (0.410–2.20)	ns
CCD1 (+ve vs. −ve)	55	3.87 (2.49–16.2)	0.001
MDM2 (−ve vs. +ve)	55	3.02 (1.54–23.7)	0.01
γ-Catenin (−ve vs. +ve)	55	3.52 (1.47–7.23)	<0.01
CCD1/MDM2 (+ve/−ve vs. −ve/+ve)	33	6.88 (1.70–39.9)	<0.05
CCD1/γ-catenin (+ve/−ve vs. −ve/+ve)	30	8.08 (4.56–46.3)	<0.0001
MDM2/γ-catenin (−ve/−ve vs. +ve/+ve)	31	4.43 (1.92–50.9)	<0.01
CCD1/MDM2/γ-catenin (+ve/−ve/−ve vs. −ve/+ve/+ve)	24	Undefined	<0.0001

LNM, lymph node metastasis; ns, not significant.

Table 4 Expression of markers related to clinicopathological parameters

	Age			*T status*			*LNM*			*Stage*		
	≧50 (%) (n = 24)	*<50 (%) (n = 31)*	*P**	*I–2 (%) (n = 45)*	*3–4 (%) (n = 10)*	*P**	*N = 0 (%) (n = 28)*	*N > 0 (%) (n = 27)*	*P**	*I–II (%) (n = 23)*	*III–IV (%) (n = 232)*	*P**
p53 (+ve)	75	83	ns	79	81	ns	75	81	ns	74	81	ns
p21 (+ve)	45	67	ns	54	72	ns	62	55	ns	57	58	ns
Rb (+ve)	75	67	ns	79	35	<0.05	78	59	ns	86	59	<0.05
CCD1 (+ve)	25	37	ns	34	30	ns	30	32	ns	26	31	ns
MDM2 (+ve)	85	90	ns	86	90	ns	85	92	ns	81	91	ns
γ-Catenin (+ve)	46	39	ns	72	36	<0.05	48	44	ns	57	26	<0.05

*Fisher's exact test; LNM, lymph node metastasis; ns, not significant.

in areca-associated OSCC (2, 3, 7). Conversely, *ras* alteration is a rare occurrence in OSCC in industrialized societies. Although BSCC is the most prevalent subset of OSCC in the areca-using population (4), its molecular alterations have been relatively unclear owing to a lower worldwide prevalence. Our previous study indicated that BSCC might preferentially carry a mutation of the p16/MTS1 gene, while OSCC of the tongue is associated with methylation in p16/MTS1 promoter region (28). The findings suggest that OSCC at different sites develops along varying pathogenetic pathways. Clusters of cell-cycle regulatory factors and cell adhesion molecular complex are good candidates for evaluating survival of BSCC (10, 20, 29–32). In this study, we identified MDM2, CCD1, and γ-catenin as markers for prognostic evaluation of areca-associated BSCC.

Several studies have shown that aberrant p53 and p21 protein expression may be correlated with the OSCC formation, although such alterations apparently do not correlate with long-term survival of OSCC (10, 20, 30). However, Chiang et al. (33) found that p53-negative OSCC had a better prognosis than p53-positive tumors. Kapranos et al. (34) showed that p21 plays a key role in the successful response to chemotherapy and may indicate a better prognosis in HNSCC. Expression of p53 and p21 in our cohort did not correlate with survival. MDM2 has been implicated in the pathogenesis of human tumors via inhibition of p53 protein function (35). However, investigation of MDM2 expression in OSCC or HNSCC has yielded mixed results (14, 17, 34). We found MDM2 expression in a high percentage of BSCC, as found in most other studies (14). However, our results suggesting that it is a favorable prognostic parameter are not in accord with other studies (14, 17). We found a lack of correlation between p53 and MDM2 expression, suggesting the existence of a p53-independent MDM2 regulatory pathway. It was also noted that a large fraction of BSCC samples displayed weak nuclear and cytoplasmic MDM2 immunoreactivity. It is likely that other elements interacting with MDM2 or multiple splicing forms of MDM2 may underlie the discrepancies of results (14).

Our data demonstrate that Rb expression was not related to the outcome of areca-associated BSCC. The reverse relationship between tumor grade and Rb expression suggests that loss of Rb expression may be a late event in oral carcinogenesis. p16/MTS1 protein elicits the dephosphorylation of Rb and thus inhibits cell cycle progression. We have previously shown the frequent abrogation of p16/MTS1 in areca-associated OSCC (28). It would be intriguing to see if there is a reciprocal relationship between Rb and p16/MTS1 expression in our cohort. It is believed that *CCD1* gene amplification is the major factor underlying protein

expression. Amplification of 11q13 where *CCD1* lies has been found to be prominent in areca-associated OSCC (36). Amplification of 11q13 has been correlated with more advanced HNSCC (37). Michalides et al. (38) found that overexpression of CCD1 is associated with more frequent recurrence of HNSCC and shortened survival. In accord with previous reports, we noted a relationship between CCD1 expression and poor survival in BSCC. We also found that CCD1 expression tended to have a predictive power equivalent to that of the LNM status (12, 17).

Downregulation of cadherin/catenin complex has been implicated in tumor progression (31). γ-Catenin expression has an inverse relationship with the degree of differentiation in OSCC. A decreased expression of γ-catenin observed at the invasive front of a carcinoma suggests a more aggressive biological behavior of these cancer cells (32). Chow et al. (39) showed that LNM status was inversely correlated with γ-catenin expression. In this study, we found that subjects with γ-catenin expression had better survival. In addition, BSCC with a larger tumor size, most extensive invasion (T4; detailed analysis not shown), and at more advanced stages had a higher incidence of the loss of γ-catenin expression.

In conclusion, this study demonstrated that BSCC with positive MDM2 and γ-catenin expression and the absence of CCD1 expression carried a better prognosis. Since the expression of these markers and the LNM status appear to be independent, their combined use could give a more accurate prediction of the patient's survival.

References

1. Chang KW, Chang CS, Lai KS, Chou MJ, Choo KB. High prevalence of human papillomavirus infection and possible association with areca quid chewing and smoking in oral epidermoid carcinomas in Taiwan. *J Med Virol* 1989; **28**: 57–61.
2. Yan JJ, Tzeng CC, Jin YT. Overexpression of p53 protein in squamous cell carcinomas of buccal mucosa and tongue in Taiwan: an immunohistochemical and clinicopathological study. *J Oral Pathol Med* 1996; **25**: 55–9.
3. Wong YK, Liu TY, Chang KW, et al. *p53* alterations in areca-quid and tobacco associated oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* 1998; **27**: 243–8.
4. Chen YK, Huang HC, Liu CM, Lin CC. Primary oral squamous cell carcinoma. an analysis of 703 cases in Southern Taiwan. *Oral Oncol* 1999; **35**: 173–9.
5. Scully C, Field JK, Tanzawa H. Genetic aberrations in oral or head and neck squamous cell carcinoma (SCCHN) 1: carcinogen metabolism, DNA repair and cell cycle control. *Oral Oncol* 2000; **36**: 256–63.
6. Scully C, Field JK, Tanzawa H. Genetic aberrations in oral or head and neck squamous cell carcinoma 2: chromosomal aberrations. *Oral Oncol* 2000; **36**: 311–27.
7. Bishop M. Molecular themes in oncogenesis. *Cell* 1991; **64**: 235–48.
8. Caamano J, Zhang SY, Rosvold EA, Bauer B, Klein-Szanto AJP. p53 alterations in human squamous cell carcinomas and carcinoma cell lines. *Am J Pathol* 1993; **142**: 1131–9.
9. De Araujo VC, Loyola AM, Santos DD, Borra RC, De Araujo NS. p53 in biopsies of oral squamous cell carcinoma. A comparative study with a malignancy grading system. *Oral Oncol* 1997; **33**: 5–9.
10. Mineta H, Borg A, Dictor M, Wahlberg P, Wennerberg J. Correlation between *p53* mutation and *cyclin D1* amplification in head and neck squamous cell carcinoma. *Oral Oncol* 1997; **33**: 42–6.
11. Agarwal S, Mathur M, Shukla NK, Ralhan R. Expression of cyclin dependent kinase inhibitor p21 in premalignant and malignant oral lesions: relationship with p53 status. *Oral Oncol* 1998; **34**: 353–60.
12. van Oijen MGCT, Tilanus MGJ, Medema RH, Slootweg PJ. Expression of p21 (Waf1/Cip1) in head and neck cancer in relation to proliferation, differentiation, p53 status and cyclin D1 expression. *J Oral Pathol Med* 1998; **27**: 367–75.
13. Chang KW, Lin SC, Kwan PC, Wong YK. Association of aberrant p53 and p21 (WAF1) immunoreactivity with the outcome of oral verrucous leukoplakia in Taiwan. *J Oral Pathol Med* 2000; **29**: 56–62.
14. Ralhan R, Sandhya A, Meera M, Bohdan W, Nootan SK. Induction of *MDM-P2* transcripts correlates with stabilized wild-type *p53* in areca- and tobacco-related human oral cancer. *Am J Pathol* 2000; **157**: 587–96.
15. Nakahara Y, Shintani S, Mihara M, Kiyota A, Ueyama Y, Matsumura T. Alterations of *Rb*, *p16 (INK 4A)* and *cyclin D1* in the tumorigenesis of oral squamous cell carcinomas. *Cancer Lett* 2000; **160**: 3–8.
16. Huang JS, Ho TJ, Chiang JP, Kok SH, Kuo YS, Kuo MY. MDM2 expression in areca quid chewing-associated oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* 2001; **30**: 53–8.
17. Millon R, Muller D, Schultz I, et al. Loss of MDM2 expression in human head and neck squamous cell carcinomas and clinical significance. *Oral Oncol* 2001; **37**: 620–31.
18. Sherr CJ, Mammalian G1 cyclins. *Cell* 1993; **73**: 1059–65.
19. Jiang W, Kahn SM, Zhou P, Zhand YJ, Infante AS. Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene* 1993; **8**: 3447–57.
20. Jares P, Fernandes PL, Campo E, Nadal A, Bosch F, Cardesa A. *PRAD-1/Cyclin D1* gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas. *Cancer Res* 1994; **54**: 4813–7.
21. Xu J, Gimenez-Conti IB, Cunningham JE, et al. Alterations of *p53*, *cyclin D1*, *Rb*, and H-*ras* in human oral carcinomas related to tobacco use. *Cancer* 1998; **83**: 204–12.
22. Yokoyama J, Shiga K, Sasano H, Suzuki M, Takasaka T. Abnormalities and the implication of retinoblastoma locus and its protein product in head and neck cancers. *Anticancer Res* 1996; **16**: 641–6.
23. Strauss M, Lukas J, Bartek J. Unrestricted cell cycling and cancer. *Nature Med* 1995; **1**: 1245–6.
24. Sofer-Leui Y, Resnitzky D. Apoptosis induced by ectopic expression of cyclin D1 but not cyclin E. *Oncogene* 1996; **13**: 2431–7.
25. Lam KY, Ng IO, Yuen AP, Kwong DL, Wei W. Cyclin D1 expression in oral squamous cell carcinomas: clinicopathological relevance and correlation with *p53* expression. *J Oral Pathol Med* 2000; **29**: 167–72.
26. Mialhe A, Louis J, Montlevier S, et al. Expression of E-cadeherin and alpha-, beta- and gamma-catenins in

human bladder carcinomas: are they good prognostic factors? *Invas Metas* 1997; **17**: 124–37.
27. Behrens J. Cadherins and catenins: role in signal transduction and tumor progression. *Cancer Metas Rev* 1999; **18**: 15–30.
28. Lin SC, Chang KW, Chang CS, et al. Alterations of p16/MTS1 gene in oral squamous cell carcinomas from Taiwanese. *J Oral Pathol Med* 2000; **29**: 159–66.
29. Kuo MYP, Lin CY, Hahn LJ, Chebg SJ, Chiang CP. Expression of cyclin D1 is correlated with poor prognosis in patients with areca quid chewing-related oral squamous cell carcinomas in Taiwan. *J Oral Pathol Med* 1999; **28**: 165–9.
30. Saito T, Nakajima T, Morgi K. Immunohistochemical analysis of cell cycle-associated proteins p16, pRb, p53, p27 and Ki-67 in oral cancer and precancer with special reference to verrucous carcinomas. *J Oral Pathol Medical* 1999; **28**: 226–32.
31. Bagutti C, Speight PM, Watt FM. Comparison of integrin, cadherin, and catenin expression in squamous cell carcinomas of the oral cavity. *J Pathol* 1998; **186**: 8–16.
32. Lo ML, Staibano S, Pannone G, et al. Beta- and gamma-catenin expression in oral squamous cell carcinomas. *Anticancer Res* 1999; **19**: 3817–26.
33. Chiang CP, Huang JS, Wang JT, Liu BY, Kuo MY. Expression of p53 protein correlates with decreased survival in patients with areca quid chewing-associated oral squamous cell carcinoma in Taiwan. *J Oral Pathol Med* 1999; **28**: 72–6.
34. Kapranos N, Stathopoulos GP, Manolopoulos L, et al. p53, p21 and p27 protein expression in head and neck cancer and their prognostic value. *Anticancer Res* 2001; **21**: 521–8.
35. Stoll C, Baretton G, Lohrs U. The influence of p53 and associated factors on the outcome of patients with oral squamous cell carcinoma. *Virchows Arch* 1998; **433**: 427–33.
36. Lin SC, Chen YJ, Hsu MD, et al. The chromosomal changes of oral squamous cell carcinoma associated with areca quid use. *Oral Oncol* 2002; **38**: 266–73.
37. Callender T, El-Nagar AK, Lee MS, et al. *PRAD-1/cyclin D1* oncogene amplification in primary head and neck squamous cell carcinoma. *Cancer* 1994; **30**: 113–20.
38. Michalides R, Van Veelen N, Hart A, Wientjens E, Balm A. Overexpression of cyclin D1 correlates with recurrence in a group of forty-seven operable squamous cell carcinomas of the head and neck. *Cancer Res* 1995; **55**: 975–8.
39. Chow V, Yuen AP, Lam KY, Tsao GS, Ho WK, Wei WI. A comparative study of the clinicopathological significance of E-cadherin and catenins (alpha, beta, gamma) expression in the surgical management of oral tongue carcinoma. *J Cancer Res Clinic Oncol* 2001; **127**: 59–63.

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