High Nuclear Protein Kinase CβII Expression Is a Marker of Disease Recurrence in Oral Squamous Cell Carcinoma

PEI-YI CHU[1], NICHOLAS CHUNG-HENG HSU[2], SHU-HUI LIN[3,4], MING-FENG HOU[5,6] and KUN-TU YEH[3,5,7]

[1]*Department of Pathology, St. Martin De Porres Hospital, Chiayi, Taiwan, R.O.C.;*
[2]*Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, R.O.C.;*
[3]*Department of Pathology, Changhua Christian Hospital, Changhua, Taiwan, R.O.C.;*
[4]*Department of Medical Technology, Jen-Teh Junior College of Medicine, Nursing and Management, Miaoli, Taiwan, R.O.C.;*
[5]*Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University Kaohsiung, Taiwan, R.O.C.;*
[6]*Cancer Center, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan, R.O.C.;*
[7]*School of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.*

Abstract. *Background: Protein kinase C beta II (PKCβII) is a member of the family of serine/threonine kinases which are involved in tumor formation and progression. This study investigated the significance of PKCβII in oral squamous cell carcinoma (OSCC). Patients and Methods: The expression of PKCβII was determined in tumors from 59 patients with OSCC using immunohistochemistry and was correlated with patients' clinical characteristics and outcomes. Results: Twenty-six cases (44%) exhibited nuclear PKCβII staining. High nuclear PKCβII expression was significantly associated with the consumption of betel quid (p=0.015) and alcohol (p=0.024) in OSCC. Kaplan–Meier analysis revealed a shorter time-to-recurrence in patients with high nuclear PKCβII expression (p=0.018). In multivariate analysis for recurrence, high nuclear staining of PKCβII remained an independent adverse prognostic factor (hazard ratio=2.3, p=0.016). Conclusion: The present study provides evidence of the potential prognostic value of PKCβII analysis in OSCC.*

Oral cancer is the fourth most common type of cancer among men in Taiwan and consists a major global public health issue and challenge (1, 2). Betel quid chewing, cigarette smoking and alcohol consumption have been identified as major etiological factors in oral cancer (3, 4). Oral squamous cell carcinoma (OSCC) represents more than 90% of all oral carcinomas. Surgery and radiation therapy remain the main treatments while chemotherapy may be used in combination with either treatment in advanced cases. The prognosis for patients with OSCC depends greatly on the stage at which the cancer is diagnosed, making early detection vital in the management of the disease.

Protein kinase C (PKC) is a family of serine/threonine kinases that participates in diverse signal transduction pathways and is involved in tumor formation and progression (5). Aberrations in PKC signaling have been implicated in the promotion and progression of many types of cancer (6-9). Among all the PKC isozymes, PKCβ is one of the most active and is involved in cell regulation (10). Two splice variants, PKCβI and PKCβII, from a single gene have been described, in which the PKCβII isozyme has been found to be involved in tumorigenesis and angiogenesis (11, 12). Differential expression of PKCβII was found among different subtypes of breast cancer (13). Overexpression of PKCβII is associated with early tumor promotion and plays a functional role in colorectal cancer (14-16). Inhibition of PKCβII induces apoptosis and suppresses the growth of human glioblastoma and colon carcinoma xenografts (17). PKCβII is also overexpressed in patients with diffuse large B-cell lymphoma and the increased expression correlates with poor prognosis (18). In this study, we investigated the expression of PKCβII in OSCC along with its clinical relevance.

Patients and Methods

Patients. Patients with OSCC who underwent surgery from May 2007 to October 2008 at the Changhua Christian Hospital, Taiwan, were investigated for PKCβII expression. Paraffin blocks were

Correspondence to: Ming-Feng Hou, MD, Cancer Center, Kaohsiung Medical University Hospital, 100 Shih-Chuan 1st Road, Kaohsiung City, Taiwan, R.O.C. Tel: +886 73164990, Fax: +886 73165011, e-mail: mifeho@kmu.edu.tw or Kun-Tu Yeh, MD, Department of Pathology, Changhua Christian Hospital, 135 Nan-Hsiao Street, Changhua, Taiwan, R.O.C. Tel: +886 47238595 ext.4830, Fax: +886 47269734, e-mail:10159@cch.org.tw

Key Words: Oral cancer, protein kinase CβII, recurrence, molecular marker, nuclear expression.

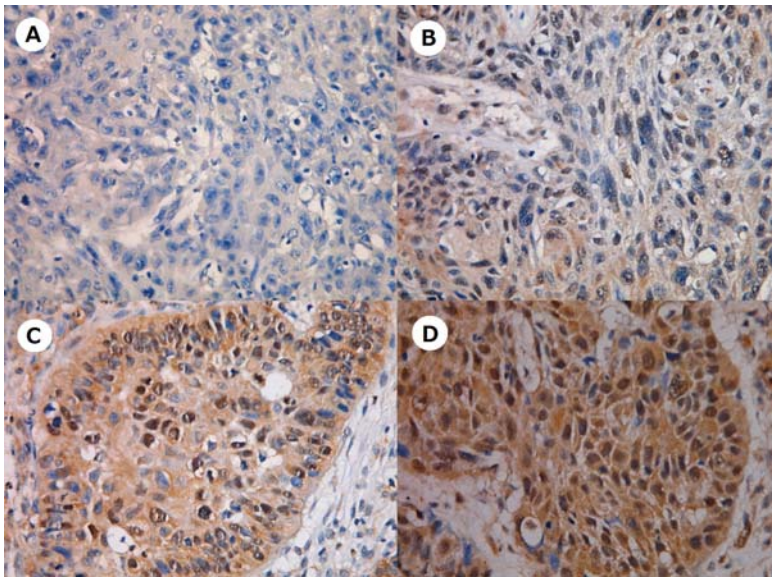

Figure 1. *Representative immunohistochemical staining patterns of oral squamous cell carcinoma for protein kinase C βII. A: Negative (–) nuclear staining; B: weak (1+) nuclear staining; C: moderate (2+) nuclear staining; and D: strong (3+) nuclear staining. Magnification 400×.*

retrospectively retrieved from the pathology archive and clinical data were obtained from medical charts and the cancer registry. None of the patients underwent radiotherapy, chemotherapy or any other treatment prior to surgery. This study was approved by the Institute Review Board at Changhua Christian Hospital, Taiwan.

Immunohistochemistry. Paraffin-embedded OSCC tissue sections (4-μm) on poly-L-lysine-coated slides were first de-waxed in xylene and rehydrated through grading solutions of alcohol, followed by a rinse using 10 mM Tris-HCl (pH 7.4) and 150 mM sodium chloride, then treated with 3% hydrogen peroxide for 5 min. Slides were incubated with an antibody against PKCβII (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min at room temperature, and thoroughly washed three times with phosphate buffered saline before incubation with an horseradish peroxidase/fragment antigen-binding polymer conjugate for another 30 min. The sites of peroxidase activity were visualized using 3,3'-diamino-benzidine tetrahydrochloride as a substrate. Gill Hematoxylin Solution II (MERCK, Darmstadt, Germany) was utilized as the counterstain. Paraffin-embedded sections of human colorectal cells of homogeneous PKCβII immunophenotype were included as positive controls. Negative controls had the primary antibody omitted and replaced by PBS. The expression of PKCβII was rated semiquantitatively based on the staining intensity. The intensity of staining was scored as –, 1+, 2+, and 3+ for negative, weak, moderate, and strong staining, respectively. Two pathologists scored coded sections independently and conflicting scores were resolved at a discussion microscope.

Statistical analysis. Correlations of PKCβII and various clinical parameters of OSCC were examined by the Pearson's chi-square test. Survival rates and disease recurrences were calculated using the Kaplan–Meier analysis and were compared by the Cochran–Mantel–Haenszel test. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death. Patients still alive at the end of the study were censored at the date of last follow-up. Time-to-recurrence was defined as the time from diagnosis until tumor recurrence or death. Statistical significance was defined as $p<0.05$.

Table I. *Differential expression of protein kinase C βII in oral squamous cell carcinoma.*

PKCβII expression	Nuclear staining/total (%)	Cytoplasmic staining/total (%)
Negative (–)	33/59 (55.9%)	3/59 (5.1%)
Weak (1+)	10/59 (16.9%)	8/59 (13.6%)
Moderate (2+)	11/59 (18.6%)	33/59 (55.9%)
Strong (3+)	5/59 (8.5%)	15/59 (25.4%)

Results

As shown in Table I, a total of 59 OSCC patients, 57 men and 2 women, were examined for the expression of PKCβII. We assessed both cytoplasmic and nuclear PKCβII expressions using a semiquantitative scoring system. Nuclear PKCβII expression with different staining intensities was noted in 26 OSCC cases. Using the semiquantitative scoring system, the OSCC patients were then classified into two groups: high-PKCβII expression with scores of 2+ or 3+, and low/negative-PKCβII expression with scores of 1+ or – (Figure 1).

High nuclear PKCβII expression was associated with alcohol consumption ($p=0.024$) and betel quid chewing ($p=0.015$) in patients with OSCC, as indicated by the Chi-

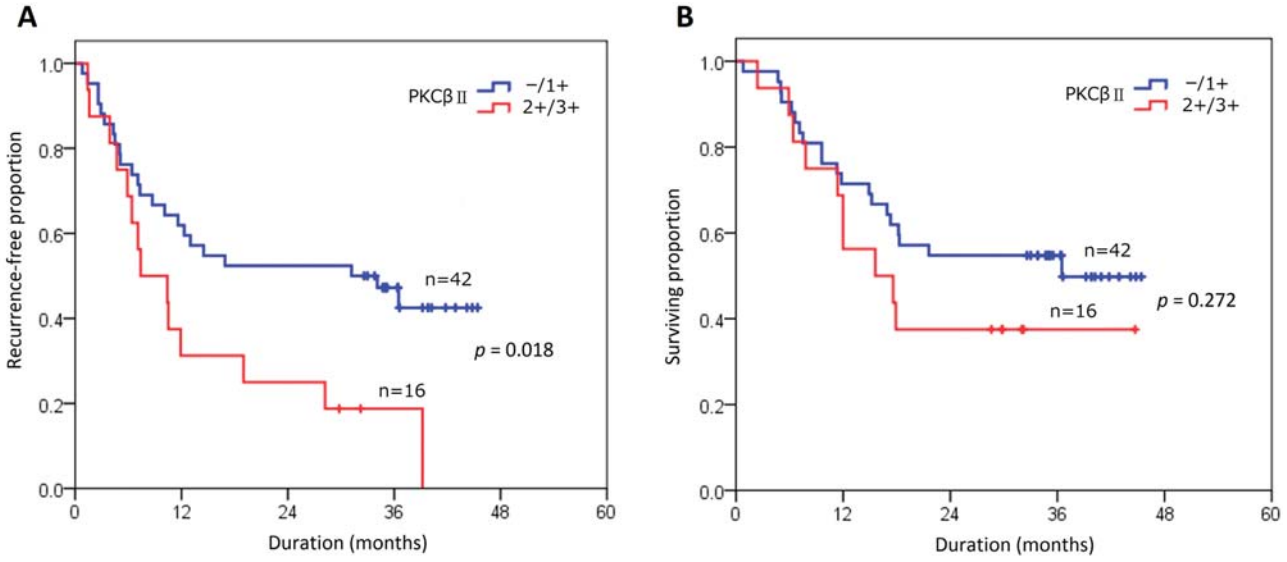

Figure 2. *The Kaplan–Meier curves of time-to-recurrence (A) and overall survival (B), stratified for protein kinase C βII phenotypes. One case lacked follow-up data and was excluded from the analysis.*

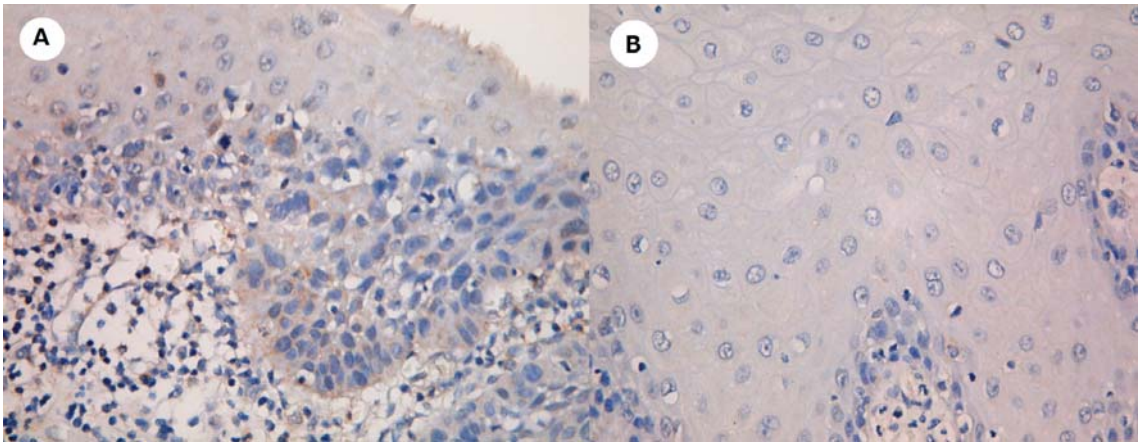

Figure 3. *Nuclear protein kinase C βII staining classified as 'negative' in dysplastic oral epithelium (A) and normal mucosal cells at the resection margin (B). Magnification 400×.*

square analyses (Table II). Follow-up data revealed that high nuclear PKCβII expression significantly correlated with tumor recurrence (p=0.018, Figure 2A). With regard to OS, the high nuclear PKCβII expression phenotype had no significant impact (Figure 2B). Cox proportional regression analyses were used to assess the effect of high nuclear PKCβII expression on tumor recurrence and OS, independently of other clinical variables. The results showed that high nuclear PKCβII expression, when adjusted for grade, tumor size, stage, and lymph node metastasis, retained a statistically significant association with disease recurrence (p=0.016, hazard ratio (HR)=2.3). Lymph node metastasis (p=0.043, HR=2.3) was the only significant risk factor with regard to survival (Table III).

Despite diffuse cytoplasmic PKCβII expression with varying levels of intensity being noted in 56/59 (95%) OSCC cases, no association was found between cytoplasmic PKCβII expression and the clinical variables studied (data not shown).

Immunohistochemical staining consistently showed a nuclear staining pattern for PKCβII to be negative in the dysplastic oral epithelium and normal mucosal cells at the resection margin (Figure 3).

Table II. *Association between nuclear protein kinase C βII expression and various clinical parameters in oral squamous cell carcinoma.*

	PKCβII expression				N	*p*-Value
	–/1+		2+/3+			
Age, years, mean (SD)	55.3 (10.6)		52.2 (14.0)		59	0.370
Alcohol consumption						
No	25	58.1%	4	25.0%	29	0.024
Yes	18	41.9%	12	75.0%	30	
Smoker						
No	16	37.2%	4	25.0%	20	0.378
Yes	27	62.8%	12	75.0%	39	
Betel chewer						
No	26	60.5%	4	25.0%	30	0.015
Yes	17	39.5%	12	75.0%	29	
Tumor grade						
I	7	16.3%	2	12.5%	9	0.711
II	31	72.1%	13	81.3%	44	
III	5	11.6%	1	6.3%	6	
Lymph node metastasis						
No	21	48.8%	8	50.0%	29	0.937
Yes	22	51.2%	8	50.0%	30	
Tumor size						
<2 cm	4	9.3%	1	6.3%	5	1.000
≥2 cm	39	90.7%	15	93.8%	54	
<4 cm	19	44.2%	8	50.0%	27	0.690
≥4 cm	24	55.8%	8	50.0%	32	
Tumor stage						
I	1	2.3%	1	6.3%	2	0.781
II	6	14.0%	1	6.3%	7	
III	7	16.3%	2	12.5%	9	
IV	29	67.4%	12	75.0%	41	

Table III. *Multivariate Cox regression analysis for the risk effect on recurrence and survival: Protein kinase C βII expression and clinicopathological risk factors.*

Risk factor	Recurrence			Survival		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
PKCβII						
–/1+	1	1.2-4.7	0.016	1	0.7-3.4	0.264
2+/3+	2.3			1.6		
Tumor grade						
I	1	0.4-2.7	0.970	1	0.3-2.4	0.692
II/III	1.0			0.8		
Lymph node metastasis						
No	1	0.7-3.0	0.290	1	1.0-5.1	0.043
Yes	1.5			2.3		
Tumor size						
<2 cm	1	0.5-11.8	0.271	1	0.2-13.5	0.610
≥2 cm	2.4			1.7		
Tumor stage						
I/II	1	0.3-2.9	0.940	1	0.5-35.1	0.167
III/IV	1.0			4.4		

HR: Hazard ratio; CI: confidence interval.

Discussion

The type of treatment for oral cancer depends on a number of factors, including size, location, type, extent of the tumor and stage of the disease. Regardless of the cause, treatment modalities in oral cancer usually involve surgery, and radiation, with or without chemotherapy. Targeted agents that cause molecular or cellular changes which are specific to oral cancer may have therapeutic potential.

Based on its role in carcinogenesis and angiogenesis, PKCβII has been suggested to represent a potential therapeutic target in many types of human cancers (11, 12). The PKC family members are well-known as shuttle molecules for cellular signaling conveying intracellular signals by moving between compartments (9). PKCs of inactive conformation reside in the cytoplasm and translocate upon stimulation to the plasma membrane and/or to the nucleus (19, 20).

Immunohistochemical staining showed the presence of PKCβII in both the cytoplasmic and nuclear compartments of OSCC cells in our study. While the pattern of cytoplasmic PKCβII expression had no clear clinical significance, high nuclear PKCβII expression was associated with alcohol and betel quid consumption (p=0.024 and 0.015, respectively). Chewing of the betel nut and alcohol consumption have both been linked to increased risk of oral cancer (3, 4). The observation that high PKCβII expression is associated with betel quid chewing and alcohol use is consistent with previous reports, which indicated that PKC expression can be induced by betel quid extracts (21), and identified PKCs as key mediators of the acute and chronic effects of alcohol (22). The Kaplan–Meier survival plots showed that OSCC patients with high nuclear PKCβII expression had a significantly shorter time-to-recurrence than patients with low/negative PKCβII expression (p=0.018). The multivariate analysis also identified the presence of PKCβII as an independent risk factor for recurrence (p=0.016). Similarly to previous epidemiological reports, we found a high male/female ratio of oral cancer incidence in this study. (23, 24).

This study is mainly limited by the rather small patient sample size for conduction of prognostic evaluation. Nonetheless, the basic characteristics are statistically well-balanced in this cohort of patients with OSCC, and the trend for shorter time-to-recurrence for patients with high nuclear PKCβII expression is clearly shown.

The present study provides, to our knowledge, the first description of PKCβII expression pattern in OSCC and its possible prognostic significance on this disease. The observation that PKCβII expression is correlated with prognosis warrants further investigation as this protein may

also represent a potential target for the therapy of OSCC. PKCβ inhibitors, such as enzastaurin, which has shown pre-clinical activity in colorectal cancer (17, 25) and glioblastoma (17), and has demonstrated clinical efficacy in diffuse large B-cell lymphoma (26), where PKCβ has been identified as a therapeutic target, might also be worthee for evaluation in OSCC.

Acknowledgements

This study was supported in part by the National Science Council (NSC-100-2321-B-750-001) and the Department of Health (DOH101-TD-C-111-002), Taiwan, R.O.C.

References

1 Fedele S: Diagnostic aids in the screening of oral cancer. Head Neck Oncol *1*: 5, 2009.
2 Yang YH, Chen CH, Chang JS, Lin CC, Cheng TC and Shieh TY: Incidence rates of oral cancer and oral pre-cancerous lesions in a 6-year follow-up study of a Taiwanese aboriginal community. J Oral Pathol Med *34*: 596-601, 2005.
3 Leite IC and Koifman S: Survival analysis in a sample of oral cancer patients at a reference hospital in Rio de Janeiro, Brazil. Oral Oncol *34*: 347-352, 1998.
4 Lo WL, Kao SY, Chi LY, Wong YK and Chang RC: Outcomes of oral squamous cell carcinoma in Taiwan after surgical therapy: Factors affecting survival. J Oral Maxillofac Surg *61*: 751-758, 2003.
5 Martiny-Baron G and Fabbro D: Classical PKC isoforms in cancer. Pharmacol Res *55*: 477-486, 2007.
6 Griner EM and Kazanietz MG: Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer *7*: 281-294, 2007.
7 Gutcher I, Webb PR and Anderson NG: The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci *60*: 1061-1070, 2003.
8 Koivunen J, Aaltonen V and Peltonen J: Protein kinase C (PKC) family in cancer progression. Cancer Lett *235*: 1-10, 2006.
9 Nishizuka Y: Protein kinase C and lipid signaling for sustained cellular responses. FASEB J *9*: 484-496, 1995.
10 Usha L, Sill MW, Darcy KM, Benbrook DM, Hurteau JA, Michelin DP, Mannel RS, Hanjani P, De Geest K and Godwin AK: A Gynecologic Oncology Group phase II trial of the protein kinase C-beta inhibitor, enzastaurin and evaluation of markers with potential predictive and prognostic value in persistent or recurrent epithelial ovarian and primary peritoneal malignancies. Gynecol Oncol *121*: 455-461, 2011.
11 Podar K, Raab MS, Chauhan D and Anderson KC: The therapeutic role of targeting protein kinase C in solid and hematologic malignancies. Expert Opin Investig Drugs *16*: 1693-1707, 2007.
12 Fields AP and Murray NR: Protein kinase C isozymes as therapeutic targets for treatment of human cancers. Adv Enzyme Regul *48*: 166-178, 2008.
13 Gokmen-Polar Y, Mehta R, Tuzmen S, Mousses S, Thorat MA, Sanders KL, Turbin D, Leung S, Huntsman DG, Sledge GW, Jr. and Badve S: Differential subcellular expression of protein kinase C βII in breast cancer: correlation with breast cancer subtypes. Breast Cancer Res Treat *124*: 327-335, 2010.
14 Gokmen-Polar Y, Murray NR, Velasco MA, Gatalica Z and Fields AP: Elevated protein kinase C βII is an early promotive event in colon carcinogenesis. Cancer Res *61*: 1375-1381, 2001.
15 Yu W, Murray NR, Weems C, Chen L, Guo H, Ethridge R, Ceci JD, Evers BM, Thompson EA and Fields AP: Role of cyclooxygenase 2 in protein kinase C βII-mediated colon carcinogenesis. J Biol Chem *278*: 11167-11174, 2003.
16 Davidson LA, Aymond CM, Jiang YH, Turner ND, Lupton JR and Chapkin RS: Non-invasive detection of fecal protein kinase C βII and ζ messenger RNA: putative biomarkers for colon cancer. Carcinogenesis *19*: 253-257, 1998.
17 Graff JR, McNulty AM, Hanna KR, Konicek BW, Lynch RL, Bailey SN, Banks C, Capen A, Goode R, Lewis JE, Sams L, Huss KL, Campbell RM, Iversen PW, Neubauer BL, Brown TJ, Musib L, Geeganage S and Thornton D: The protein kinase Cβ-selective inhibitor, enzastaurin (LY317615.HCl), suppresses signaling through the AKT pathway, induces apoptosis, and suppresses growth of human colon cancer and glioblastoma xenografts. Cancer Res *65*: 7462-7469, 2005.
18 Shipp MA, Ross KN, Tamayo P, Weng AP, Kutok JL, Aguiar RC, Gaasenbeek M, Angelo M, Reich M, Pinkus GS, Ray TS, Koval MA, Last KW, Norton A, Lister TA, Mesirov J, Neuberg DS, Lander ES, Aster JC and Golub TR: Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. Nat Med *8*: 68-74, 2002.
19 Newton AC: Protein kinase C: Structural and spatial regulation by phosphorylation, co-factors, and macromolecular interactions. Chem Rev *101*: 2353-2364, 2001.
20 Martelli AM, Sang N, Borgatti P, Capitani S and Neri LM: Multiple biological responses activated by nuclear protein kinase C. J Cell Biochem *74*: 499-521, 1999.
21 Lee MJ, Hsu JD, Lin CL, Lin MH, Yuan SC and Wang CJ: Induction of epidermal proliferation and expressions of PKC and NF-κB by betel quid extracts in mouse: The role of lime-piper additives in betel quid. Chem Biol Interact *140*: 35-48, 2002.
22 Newton PM and Ron D: Protein kinase C and alcohol addiction. Pharmacol Res *55*: 570-577, 2007.
23 Chiang CT, Chang TK, Hwang YH, Su CC, Tsai KY, Yuan TH and Lian Ie B: A critical exploration of blood and environmental chromium concentration among oral cancer patients in an oral cancer-prevalent area of Taiwan. Environ Geochem Health *33*: 469-476, 2011.
24 Ko YC, Huang YL, Lee CH, Chen MJ, Lin LM and Tsai CC: Betel quid chewing, cigarette smoking and alcohol consumption related to oral cancer in Taiwan. J Oral Pathol Med *24*: 450-453, 1995.
25 Fields AP, Calcagno SR, Krishna M, Rak S, Leitges M and Murray NR: Protein kinase Cβ is an effective target for chemoprevention of colon cancer. Cancer Res *69*: 1643-1650, 2009.
26 Robertson MJ, Kahl BS, Vose JM, de Vos S, Laughlin M, Flynn PJ, Rowland K, Cruz JC, Goldberg SL, Musib L, Darstein C, Enas N, Kutok JL, Aster JC, Neuberg D, Savage KJ, LaCasce A, Thornton D, Slapak CA and Shipp MA: Phase II study of enzastaurin, a protein kinase C β inhibitor, in patients with relapsed or refractory diffuse large B-cell lymphoma. J Clin Oncol *25*: 1741-1746, 2007.

Received May 2, 2012
Revised July 17, 2012
Accepted July 18, 2012