Significant Association of *Cyclin D1* Single Nucleotide Polymorphisms with Oral Cancer in Taiwan

MING-HSUI TSAI[1,4,8], CHIA-WEN TSAI[4,5,8], YUNG-AN TSOU[1,4,8], CHUN-HUNG HUA[1,4], CHIA-FANG HSU[4,7], and DA-TIAN BAU[4,5,6],

[1] *Departments of Otolaryngology,* [2] *Hematology Oncology,* [3] *Pediatrics,* [4] *Terry Fox Cancer Research Lab, China Medical University Hospital, Taichung, Taiwan, R.O.C.*

[5] *Graduate Institute of Basic Medical Science,* [6] *Graduate Institutes of Clinical Medical Science, China Medical University, Taichung, Taiwan, R.O.C.*

[7] *Department of Otolaryngology, Chang-Hua Hospital, Chang-Hua, Taiwan, R.O.C*

[8] *These authors contribute equally to this work*

Correspondence to: Da-Tian Bau Ph.D., Terry Fox Cancer Research Lab, Department of Medical Research, China Medical University Hospital, 2 Yuh-Der Road, Taichung, 404 Taiwan, R.O.C. Tel: +886422053366 Ext 3312, Fax: +886422053366 Ext 1511, e-mail: datian@mail.cmuh.org.tw; artbau1@yahoo.com.tw

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Abstract. Aim: The cell cycle regulator *cyclin D1 (CCND1)* is thought to play a major role in the transition of cell cycle from G1 to S phase. It is known that cancer cells have an unbalanced cell cycle regulation. The study aimed at investigating the association of *CCND1* and examining the gene-environment interaction among *CCND1* and smoking habits. **Materials:** In this hospital-based case-control study, the associations of *CCND1* single nucleotide polymorphisms A870G (rs9344) and C1722G (rs678653) with oral cancer risk were investigated in 620 patients and 620 age-and gender-matched controls. **Results:** The results confirmed that there were significant differences between oral cancer and control groups in the distribution of the genotypes (*P*=0.0014) and allelic frequency (*P*=0.0027) in the *CCND1* rs9344 genotype. Individuals who carried at least one G allele (GG or AG) had a 0.64-fold increased risk of developing oral cancer compared to those who carried the AA wild type genotype (95% CI: 0.50-0.81). There are obvious joint effects of *CCND1* rs9344 genotypes with smoking habits on oral cancer. **Conclusion:** These findings support the conclusion that the cell cycle regulation may play a role in oral carcinogenesis and that *CCND1* rs9344 polymorphism maybe a useful biomarker for oral oncology.

Key Words: Cyclin D1, polymorphism, oral cancer, smoking.

Oral cancer is commonly diagnosed cancers all over the world (1-4). With continuously increasing incidence and mortality for the past two decades, oral cancer has become the fourth most common cause of male cancer death in Taiwan. (5). Smoking may induce oxidative insults to the human genome, with the major DNA adducts of 8-hydroxy-2-deoxyguanine (8-OH-dG) (6, 7). The 8-OH-dG is mutagenic which if not repaired on time, can cause severe transversions of GC to TA in several oncogenes and tumor suppressor genes and in turn lead to carcinogenesis (6, 7). Thus, smoking habit is one of the environmental factors for oral oncology.

Cyclin D1 (CCND1) plays a critical role in the G1-S phase transition of the cell cycle (8, 9). CCND1 accomplishes this key function by forming complex with its kinase partners CDK 4 or CDK6 (8, 9). Some reports has demonstrated CCND1 may involved in the development of some cancers in a CDK-independent pattern (10, 11). Dysregulation of CCND1 is a commonly observed character of human cancers, and frequently an overexpression of CCND1 has been reported as a potential biomarker in human cancers, such as oral carcinoma (12-14). However, the underlying mechanisms of the CCND1 overexpression and its relationship to oral oncology is poorly understood. In the literature, limited information is available of the genetic role of *CCND1* in oral cancer, except on in head and neck cancer (15), one in oral premalignant lesion(16), and two in oral cancer (17, 18). In this study, we aimed at

evaluating the contribution of *CCND1* polymorphisms to oral cancer in Taiwan. In addition, we also investigated the genotype joint interaction with smoking behaviors.

Materials and Methods

Study population and sample collection. Six hundred and twenty cancer patients diagnosed with oral cancer were recruited at the outpatient clinics of general surgery between 1998-2010 at the China Medical University Hospital, Taichung, Taiwan. The clinical characteristics of patients including histological details were all graded and defined by expert surgeons. All patients voluntarily participated, completed a self-administered questionnaire and provided peripheral blood samples. As many non-oral cancer healthy volunteers as controls were selected by matching for age, gender and habits after initial random sampling from the Health Examination Cohort of the hospital. The exclusion criteria of the control group included previous malignancy, metastasized cancer from other or unknown origin, and any familial or genetic diseases. Both groups completed a short questionnaire which included habits. Smokers were defined as daily or almost daily smokers, who had smoked at least five packs of cigarettes in their lifetime. Smokers were asked for the age of initiation, whether they were currently smoking or had already quit, and if so, when they had quit, and on average, how many cigarettes they smoked or had smoked daily. Our study was approved by the Institutional Review Board of the China Medical

University Hospital and written-informed consent was obtained from all participants.

Genotyping conditions. Genomic DNA was prepared from peripheral blood leucocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and stored as previously published (19-24). The primers used for *CCND1* A870G (rs9344) were: forward 5'-GTG AAG TTC ATT TCC AAT CCG C-3', and reverse 5'-GGG ACA TCA CCC TCA CTT AC-3'; for *CCND1* C1722G (rs678653) were: forward 5'-CTC TTG GTT ACA GTA GCG TAG C-3', and reverse 5'-ATC GTA GGA GTG GGA CAG GT-3'. The following cycling conditions were performed: one cycle at 94^{o}C for 5 min; 35 cycles of 94^{o}C for 30 s, 55^{o}C for 30 s, and 72^{o}C for 30 s; and a final extension at 72^{o}C for 10 min.

RFLP conditions. As for the *CCND1* rs9344, the resultant 167 bp PCR product was mixed with 2 U *Nci* I and incubated for 3 h at 37°C. The G form PCR products could be further digested while the A form could not. Two fragments 145 bp and 22 bp were present if the product was digestible G form. As for the *CCND1* rs678653, the resultant 159 bp PCR product was mixed with 2 U *Hae* III and incubated for 3 h at 37°C. On digestion with *Hae* III, the PCR product arising from the G allele was cut into fragments of 111, 26 and 22 bp, whereas C allele was cut into fragments of 137 and 22 bp. Then, 10 μl of product was loaded into a 3% agarose gel containing

ethidium bromide for electrophoresis. The genotype analysis was performed by two researchers independently and blindly. Ten percent of the samples were randomly selected for direct sequencing and the results were 100% concordant.

Statistical analyses. To ensure that the controls used were representative of the general population and to exclude the possibility of genotyping error, the deviation of the genotype frequencies of *CCND1* single nucleotide polymorphisms in the control subjects from those expected under the Hardy-Weinberg equilibrium was assessed using the goodness-of-fit test. Pearson's Chi-square test or Fisher's exact test (when the expected number in any cell was less than five) was used to compare the distribution of the *CCND1* genotypes between cases and controls. Cancer risk associated with the genotypes was estimated as odds ratio (ORs) and 95% confidence intervals (CIs) using unconditional logistic regression. Data was recognized as significant when the statistical *P*-value was less than 0.05.

Results

There were no significant differences between both groups in their age, sex, and smoking status (Table I). The frequencies of the genotypes and alleles of the *CCND1* A870G (rs9344) in the oral cancer and control groups were shown in Table II. There

were significant differences between both groups in the distribution of genotype (*P*=0.0014) and allelic frequency (*P*=0.0027). The odds ratios of the AG and GG were 0.64 (95%CI=0.50-0.81) and 0.61 (95%CI=0.43-0.87), respectively, compared to the AA wild-type genotype. Hence, individuals who carried at least one G allele (AG and GG) had a 0.64-fold increased risk of developing oral cancer compared to those who carried the A/A wild type genotype (95%CI: 0.50-0.81). Allele G had a 0.78-fold increased risk of developing oral cancer compared to allele A (95%CI: 0.67-0.92). On the contrary, as for the *CNND1* C1722G, there was no difference in the distributions of either genotype or allelic frequency between oral cancer patient and control groups (Table III). The conclusive finding deduced from the data in Tables II and III is that the G allele of *CNND1* A870G seems to be protective factor for oral cancer in Taiwan.

The interaction of genotype of *CNND1* A870G and the smoking habits was of our interest. The genotype distribution of various genetic polymorphisms of *CNND1* A870G was significantly different between oral cancer and control groups who have smoking habit (*P*=0.0006) (Table IV). Consistent with the findings in Table II, the GG genotype frequency was still significantly lower (12.9%) in cancer patients who have smoking habit than in smoking controls (16.6%). There was no such distribution difference in the non-smoking groups (*P*>0.05).

Discussion

In order to examine the role of *CNND1* in oral cancer, in this study, we selected the most commonly studied two polymorphic sites of the *CNND1* gene, A870G (rs9344) and C1722G (rs678653), and clarify their associations with the susceptibility for the oral cancer risk in Taiwan, where the oral cancer density is the highest worldwide. We found that the G variant genotypes of *CNND1* A870G were significantly associated with a lower susceptibility for oral cancer (Tables II), and this genotype had joint effects with individual smoking habits on oral cancer susceptibility (Table IV), while the *CNND1* C1722G polymorphism may play a minor role in oral carcinogenesis. As we supposed, the effects of *CNND1* gene on oral carcinogenesis are complex, exerting either an adverse effect or an advantageous influence on determining oral cancer risk.

Several studies showed that the genotypes of *CNND1* A870G were associated with cancer risks, however which genotype plays more critical remains unclear and it is quite disease- and ethic-dependent. Consistent with our findings, the G allele seems to be protective factor in hepatocellular carcinoma (25), larynx (26), breast (27), colorectal (28, 29), and bladder cancers (30). But several controversial findings reported that the G allele was risky in oral (18) and colorectal cancer (31), or not associated in oral (17, 32) and other cancers (33-35). Among the studies, the sample

sizes all needed to be enlarged and certificated, and a conclusion of the genetic role of CNND1 play in carcinogenesis are still not easily deduced so far.

In literature, the overexpression of CNND1 were found to be associated with oral cancer risk (12, 36). However, the underlying mechanism leading to this aberrant expression remains poorly understood. One of the probable mechanisms of CNND1 overexpression is alternate splicing modulated by A870G (37, 38) to sustain the protein for a longer time. Recently, in esophageal adenocarcinomas, The A allele of A870G was found to promote cyclin D1 expression (39). Contradictory to the study, also performed in head and neck cancer, no association between A870G polymorphism and cyclin D1 expression was reported (39). Therefore, the genotype-phenotype correlation, and their relation to oral oncology need to be further conformed in the future.

To sum up, this is so far the largest study which focuses on the *CNND1* and its joint effects with smoking habit on oral cancer risk. The genotype of *CNND1* A870G, interacts with smoking habits, may play an important role in the oral carcinogenesis.

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Table I. Age, gender and cigarette smoking status of oral cancer patients and controls

Characteristics	Controls (n = 620)			Patients (n = 620)			*P*[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (y)			51.3 (7.4)			52.4 (7.2)	0.78
Gender							1.00
Male	586	94.5%		586	94.5%		
Female	34	5.5%		34	5.5%		
Cigarette smoking							
Yes	443	71.5%		458	73.9%		0.37
No	177	28.5%		162	26.1%		

Table II. Distribution of *CCND1* A870G (rs9344) genetic and allelic frequencies among oral cancer patient and control groups.

A870G (rs9344)	Controls	%	Patients	%	OR (95% CI)[a]	*P-value*[b]
Genetic frequency						
AA	155	25.0%	213	34.4%	1.00 (Reference)	0.0014
AG	365	58.9%	323	52.1%	**0.64 (0.50-0.83)**	
GG	100	16.1%	84	13.5%	**0.61 (0.43-0.87)**	
Carrier comparison						
AA+AG	520	83.9%	536	86.5%	1.00 (Reference)	NS
GG	100	16.1%	84	13.5%	0.81 (0.60-1.12)	
AA	155	25.0%	213	34.4%	1.00 (Reference)	0.0004
AG+GG	465	75.0%	407	65.6%	**0.64 (0.50-0.81)**	

Allele frequency						
Allele A	675	54.4%	749	60.4%	1.00 (Reference)	0. 0027
Allele G	565	45.6%	491	39.6%	**0.78 (0.67-0.92)**	

[a] OR: odds ratio, CI: confidence interval; [b] Based on Chi-square test, NS: non-significant.

Table III. Distribution of *CCND1* C1722G (rs678653) genetic and allelic frequencies among oral cancer patient and control groups.

C1722G (rs678653)	Controls	%	Patients	%	OR (95% CI)[a]	*P-value*[b]
Genetic frequency						
GG	434	70.0%	450	72.6%	1.00 (Reference)	NS
CG	136	21.9%	127	20.5%	0.90 (0.68-1.19)	
CC	50	8.1%	43	6.9%	0.83 (0.54-1.27)	
Carrier comparison						
GG+CG	570	91.9%	577	93.1%	1.00 (Reference)	NS
CC	50	8.1%	43	6.9%	0.85 (0.56-1.30)	
GG	434	70.0%	450	72.6%	1.00 (Reference)	NS
CG+CC	186	30.0%	170	27.4%	0.88 (0.69-1.13)	

Allele frequency						
Allele G	1004	81.0%	1027	82.8%	1.00 (Reference)	NS
Allele C	236	19.0%	213	17.2%	0.88 (0.72-1.08)	

[a] OR: odds ratio, CI: confidence interval; [b] Based on Chi-square test, NS: non-significant.

Table IV. Distribution of *CCND1* A870G (rs9344) genotypes in oral cancer patients after stratification by cigarette smoking habits.

Variable	*CCND1* A870G (rs9344) genotype			
	AA (%)	AG (%)	GG (%)	*P*-value[a]
Smokers				
Controls	100 (23.1%)	261 (60.3%)	72 (16.6%)	**0.0006**[b]
Patients	159 (34.7%)	240 (52.4%)	59 (12.9%)	
Non-smokers				
Controls	55 (29.4%)	104 (55.6%)	28 (15.0%)	NS
Patients	54 (33.3%)	83 (51.2%)	25 (15.5%)	

[a] Based on Chi-square test, NS: non-significant.