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

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Abstract. *The cell cycle regulator cyclin D1 (CCND1) is thought to play a major role in the transition of the cell cycle from G_1 to S-phase. It is known that cancer cells have unbalanced cell cycle regulation. This study aimed to investigate the association of CCND1 single nucleotide polymorphisms A870G (rs9344) and C1722G (rs678653) with oral cancer risk and examine the interaction between CCND1 and smoking habit. Materials and Methods: In this hospital-based case–control study, the CCND1 polymorphisms were investigated in 620 patients and 620 age- and gender-matched controls. Results: Significant differences were shown between the 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 decreased risk of developing oral cancer compared to those who carried the AA wild-type genotype (95% CI: 0.50-0.81). There was an obvious joint effect of CCND1 rs9344 genotype with smoking habit on oral cancer. Conclusion: Cell cycle regulation may play a role in oral carcinogenesis and CCND1 rs9344 polymorphism maybe a useful biomarker for oral oncology.*

Oral cancer is one of the commonly diagnosed carcinomas throughout 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) the highest figure worldwide. Smoking may induce oxidative insults to the human genome, with the major DNA adduct of 8-hydroxy-2-deoxyguanine (8-OH-dG) (6, 7). The 8-OH-dG is mutagenic and, if not repaired in 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, a smoking habit is one of the lifestyle factors for oral oncology.

Cyclin D1 (CCND1) plays a critical role in the G_1 to S-phase transition of the cell cycle (8, 9). CCND1 accomplishes this key function by forming a complex with its kinase partners cyclin dependent kinase (CDK) 4 or CDK6 (8, 9). Some reports have demonstrated that CCND1 may be involved in the development of some carcinomas in a CDK-independent pattern (10, 11). Dysregulation of CCND1 is a commonly observed character of human carcinomas and frequently an overexpression of CCND1 has been reported as a potential biomarker in human carcinomas, such as oral carcinoma (12-14). However, the underlying mechanisms of CCND1 overexpression and its relationship to oral oncology are poorly understood. In the literature, information regarding the genetic role of *CCND1* in oral cancer is limited, with one study in head and neck cancer (15) and in oral premalignant lesions (16), and two in oral cancer (17, 18). In this study, the contribution of A870G (rs9344) and C1722G (rs678653), the two most commonly studied *CCND1* polymorphisms, to oral cancer in Taiwan was evaluated. In addition, the genotype interaction with smoking behavior was also investigated.

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 and 2010 at the China Medical University Hospital, Taichung, Taiwan. The clinical

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characteristics of the patients including histological details were all graded and defined by expert surgeons. All the 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. The 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. The study was approved by the Institutional Review Board of the China Medical University Hospital and written-informed consent was obtained from all the 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'and 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˚C for 5 min; 35 cycles of 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s; and a final extension at 72˚C for 10 min.

Restriction fragment length polymorphism (RFLP) conditions. For *CCND1* rs9344, the 167 bp PCR product was mixed with 2 U *Nci I* and incubated for 3 h at 37˚C. The G form PCR products were further digested while the A form was not. Two fragments 145 bp and 22 bp were present if the product was the digestible G form. For *CCND1* rs678653, the 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 the C allele was cut into fragments of 137 and 22 bp. Then, 10 μl of each 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.

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

Characteristic	Controls (n=620)			Patients (n=620)			*P*[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			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		

Results

There were no significant differences between the oral cancer patients and the controls 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 are shown in Table II. There were significant differences between the groups in the distribution of genotype (p=0.0014) and allelic frequency (p=0.0027). The ODs of the AG and GG were 0.64 (95% CI=0.50-0.83) 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 decreased risk of developing oral cancer compared to those who carried the AA wild-type genotype. Allele G conferred a 0.78-fold decreased risk of developing oral cancer compared to allele A. In contrast, for *CNND1* C1722G, there was no difference in the distributions of either genotype or allelic frequency between the oral cancer patient and control groups (Table III).

The genotype distribution of the polymorphisms of *CNND1* A870G was significantly different between the oral cancer patients and the controls who had a 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 the cancer patients who had a smoking habit than in the smoking controls (16.6%). There was no such distribution difference in the non-smoking groups (p>0.05).

Discussion

The conclusive finding deduced from the data in Table II is that the G allele of *CNND1* A870G seems to be a protective factor for oral cancer in Taiwan. The G variant genotypes of *CNND1* A870G were significantly associated with a lower susceptibility for oral cancer, and this genotype had a joint effect with smoking habit on oral cancer susceptibility (Table IV), while the *CNND1* C1722G polymorphism may play a

Table II. *Distribution of CCND1 A870G (rs9344) genetic and allelic frequencies in the oral cancer patients and control groups.*

A870G (rs9344)	Controls	%	Patients	%	OR (95% CI)	*P*-value[a]
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)**	

OR: Odds ratio, CI: confidence interval, [a]based on Chi-square test, NS: non-significant.

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

C1722G (rs678653)	Controls	%	Patients	%	OR (95% CI)	*P*-value[a]
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)	

OR: Odds ratio, CI: confidence interval, [a]based on Chi-square test, NS: non-significant.

minor role in oral carcinogenesis. 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 have shown that the genotypes of *CNND1* A870G were associated with cancer risk, however which genotype plays a more critical role remains unclear and is quite disease- and ethnic-dependent. Consistent with the present findings, the G allele seemed to be a protective factor in hepatocellular carcinoma (25), laryngeal (26), breast (27), colorectal (28, 29) and bladder carcinomas (30). But several contrasting findings that the G allele was a risk in oral (18) and colorectal cancer (31), or not associated in oral (17, 32) and other carcinomas (33-35) have been reported. Among the studies, the sample sizes all needed to be larger and a resolution of the genetic role of *CNND1* in carcinogenesis is still not easily achieved.

In the literature, the overexpression of CNND1 was 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). Contradictorily, also investigated in head and neck cancer, no association between A870G polymorphism and cyclin D1 expression was reported (39). Therefore, the genotype-phenotype correlation, and the relation to oral oncology need to be further confirmed.

This was to date the largest study which focused on *CNND1* and its joint effect with the smoking habit on oral cancer risk. The A870G genotype of *CNND1* interacts with smoking habit and may play an important role in oral carcinogenesis.

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

Variable	CCND1 A870G (rs9344) genotype			
	AA (%)	AG (%)	GG (%)	*P*-value[a]
Smokers				
Controls	100 (23.1)	261 (60.3)	72 (16.6)	**0.0006**
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.

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