

Association of *Cyclin D1* Genotypes with Nasopharyngeal Carcinoma Risk

Liang-Chun Shih^{1,2*}, Chia-Wen Tsai^{2,3*}, Ming-Hsui Tsai^{1,2*}, Yung-An Tsou^{1,2}, Wen-Shin Chang^{2,3}, Fang-Jing Li², Meng-Hsuan Lee^{2,4} and Da-Tian Bau^{2,3,4}

¹ *Department of Otolaryngology and* ² *Terry Fox Cancer Research Laboratory, China Medical University Hospital, Taichung, Taiwan, R.O.C.;*

³ *Graduate Institute of Basic Medical Science,* ⁴ *Graduate Institute of Clinical Medical Science, China Medical University, Taichung, Taiwan, R.O.C.*

Correspondence to: Da-Tian Bau Ph.D., Terry Fox Cancer Research Laboratory, 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; artbau2@gmail.com

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* These authors contributed equally to this study.

Abstract. Aim: The cell cycle regulator *cyclin D1* (*CCND1*) is a critical regulator of the G₁/S phase transition and plays an important part in several tumor types. This study aimed at investigating the association of *CCND1* with and examining the interaction among *CCND1* genotype and individual smoking habit in nasopharyngeal carcinoma susceptibility. **Materials and Methods:** A total of 352 native Taiwanese consisting of 176 cases and 176 controls were enrolled in this hospital-based study, and *CCND1* A870G (rs9344) and C1722G (rs678653) genotyping were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and partially verified by direct sequencing. **Results:** The results showed that there were significant differences between nasopharyngeal carcinoma and control groups in the distribution of the genotypic ($P=0.0222$) and allelic ($P=0.0322$) frequencies in the *CCND1* A870G genotype. Individuals who carried at least one G allele (GG or AG) had a 0.71-fold lower risk of developing nasopharyngeal carcinoma compared to those who had the AA genotype (95% confidence interval=0.53-0.96). In addition, there is an obvious joint effect of *CCND1* A870G genotype with smoking habit on nasopharyngeal carcinoma susceptibility. **Conclusion:** These findings support the conclusion that the cell cycle regulation may play a role in nasopharyngeal carcinoma development and that *CCND1* A870G polymorphism maybe a useful biomarker for nasopharyngeal carcinoma progression.

Key Words: Cyclin D1, polymorphism, nasopharyngeal carcinoma, smoking habit.

Nasopharyngeal carcinoma is the cancer originating in the nasopharynx, the uppermost region of the pharynx, behind the nose where the nasal passages and auditory tubes join the remainder of the upper respiratory tract. Geographically, nasopharyngeal carcinoma occurs sporadically in the West, and more common in certain regions of Asia than elsewhere, for instance, Southern China (age standardized rate=30-50/100,000) Southeast Asia (age standardized rate=9-12/100,000) and Taiwan (age standardized rate=8.2-8.4/100,000) (1-3). The geographical pattern of nasopharyngeal carcinoma incidence suggests a unique interaction of Epstein-Barr virus (EBV) viral, dietary and genetic factors (4-9).

Nasopharyngeal carcinoma has been thought to be one of the smoking-related cancers. In literature, smoking has been found to induce oxidative insults to the human genome, with the major DNA adducts of 8-hydroxy-2-deoxyguanine (8-OH-dG) (10, 11). 8-OH-dG is mutagenic, and, if not repaired in time, can cause severe GC to TA transversions in several oncogenes and tumor suppressor genes and, in turn, lead to carcinogenesis (10, 11).

Cyclin D1 (CCND1) plays a critical role in the G₁/S phase transition of the cell cycle (12), which accomplishes this critical role by forming a complex with its kinase partners CDK 4 or CDK6 (12, 13). Some reports have demonstrated that CCND1 may be involved in the development of some types of tumors in a CDK-independent

pattern (14, 15). Dysregulation of CCND1 is a commonly observed characteristic of human cancer, and frequently overexpression of CCND1 has been reported as a potential biomarker in human cancer, such as oral carcinoma (16-18). However, the underlying mechanisms of CCND1 overexpression and its relationship to nasopharyngeal carcinoma progression are poorly understood. In the literature, limited information is available regarding the genetic role of *CCND1* in nasopharyngeal carcinoma, with only some articles on head and neck cancer (19), in oral premalignant lesion (20), and two on oral cancer (21, 22).

In this study, we aimed at evaluating the contribution of *CCND1* polymorphisms to nasopharyngeal carcinoma in Taiwan. In addition, we also investigated the joint interaction of genotype with smoking behaviors.

Materials and Methods

Study population and sample collection. One hundred and seventy-six cancer patients diagnosed with nasopharyngeal carcinoma were recruited at the outpatient clinics of general surgery between 2003-2009 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 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 recorded for their age of smoking 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 leukocytes using a QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) and stored as previously published (23-29). The primers used for *CCND1* A870G 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 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 used: 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* A870G, 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 of 145 bp and 22 bp were present if the product was the digestible G form. As for the *CCND1* C1722G, 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. Subsequently, 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. Data was recognized as significant when the statistical *P*-value was less than 0.05.

Results

The characteristics of nasopharyngeal carcinoma patients and the non-cancer controls are summarized in Table I. There were no significant differences between the groups in their age, sex, cigarette smoking, betel quid chewing and alcohol drinking status (Table I). The frequencies of the genotypes and alleles of the *CCND1* A870G in the nasopharyngeal carcinoma and control groups are shown in Table II. Firstly, there was a significant difference between nasopharyngeal carcinoma and control groups in the distribution of genotypic frequency ($P=0.0222$), and the odds ratio of the AG was 0.59 (95% confidence interval, CI=0.36-0.94), compared to the AA wild-type genotype. Secondly, we have performed the dominant and recessive comparison, finding that the odds ratios of the AG+GG *versus* AA were 0.42 (95% CI=0.26-0.68, $P=0.0080$). Lastly, there was also a significant difference between nasopharyngeal carcinoma and control groups in the distribution of allelic frequency ($P=0.0322$). To sum up, individuals who carried at least one G allele (AG and GG) had a lower risk of

developing nasopharyngeal carcinoma compared to those who carried the AA wild-type genotype (Table II). As for the *CCND1* C1722G, there was no difference in the distributions of either genotype or allelic frequency between nasopharyngeal carcinoma patient and control groups (Table III). The conclusive finding deduced from the data in Tables II and III is that the G allele of *CCND1* A870G seems to be a protective factor for nasopharyngeal carcinoma in Taiwan.

The interaction of the genotype of *CCND1* A870G and cigarette smoking, betel quid chewing, and alcohol drinking habits was of interest. The genotype distribution of various genetic polymorphisms of *CCND1* A870G was significantly different between nasopharyngeal carcinoma and control groups who have smoking habit ($P=0.0165$) (Table IV). Consistent with the findings in Table II, the GG genotype frequency was still significantly lower (6.5%) in cancer patients who have smoking habit than in smoking controls (15.0%). There was no such distribution difference in the non-smoking groups ($P>0.05$). There was no obvious interaction between the genotype of *CCND1* A870G with individual betel quid chewing, and alcohol drinking habits found (data not shown).

Discussion

In order to examine the role of *CCND1* in nasopharyngeal carcinoma, in this study,

we selected two common polymorphic sites of the *CCND1* gene, A870G (rs9344) and C1722G (rs678653), and clarified their associations with susceptibility for nasopharyngeal carcinoma risk in central Taiwan. We found that the G variant genotypes of *CCND1* A870G were significantly associated with a lower susceptibility for nasopharyngeal carcinoma (Table II), and this genotype had joint effects with individual smoking habits on nasopharyngeal carcinoma susceptibility (Table IV), while the *CCND1* C1722G genotype may contribute very little to susceptibility to nasopharyngeal carcinoma. At the same time, no obvious joint effect of *CCND1* C1722G genotype with either betel quid chewing or alcohol drinking habits on nasopharyngeal carcinoma risk was found.

Several studies showed that the genotypes of *CCND1* A870G were associated with cancer risk, however, which genotype plays the more critical role remains unclear and it may be quite disease- and ethnicity-dependent. Consistent with our results in nasopharyngeal carcinoma, the G allele seems to be a protective factor in hepatocellular carcinoma (30), laryngeal (31), breast (32), colorectal (33, 34), and bladder tumors (35). But several controversial findings reported that the G allele was risky in oral (22) and colorectal cancer (36), or not associated in various types of cancer (21, 37-40). There is no denying that the investigated sample sizes of all these studies needed to be enlarged and their finding confirmed by other teams; any

conclusion of the genetic role that *CCND1* plays in carcinogenesis can still not easily be made.

From the proteomic viewpoint, the overexpression of *CCND1* was found to be associated with oral cancer risk (16, 41). However, the underlying mechanism leading to this aberrant expression remains poorly understood. One of the probable mechanisms of *CCND1* overexpression is alternate splicing modulated by A870G, which can lead to the sustainment of the *CCND1* protein for a longer time (42, 43). Recently, in esophageal adenocarcinomas, the A allele of A870G was found to promote *CCND1* expression (44). Contradictory to this study, also performed in head and neck cancer, no association between A870G polymorphism and *CCND1* expression was reported in another study (44). The limited sample size of this study leads to some borderline findings (Table II, III and IV), and should be verified soon in the near future with an enlarged population. The genotype–phenotype correlation and detailed mechanisms of *CCND1* contributing to nasopharyngeal carcinoma need further investigations.

To sum up, this is so far the earliest study which focuses on *CCND1* and its joint effects with smoking habit on nasopharyngeal carcinoma risk. The genotype of *CCND1* A870G, interacting with smoking habit, may play an important role in the progression of nasopharyngeal carcinoma.

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Table I. Characteristics of nasopharyngeal carcinoma patients and controls.

Characteristic	Controls (n = 176)			Patients (n = 176)			P-value ^a
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			49.3 (9.4)			48.2 (11.1)	0.6851
Gender							1.0000
Male	128	72.7%		128	72.7%		
Female	48	27.3%		48	27.3%		
Indulgence							
Cigarette smoking	73	41.5%		77	43.8%		0.7465
Betel quid chewing	54	30.7%		55	31.3%		1.0000
Alcohol drinking	72	40.9%		80	45.5%		0.5414

^a Based on Chi-square test.

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

A870G (rs9344)	Controls		Patients		OR (95% CI)	P-value ^a
	n	%	n	%		
Genetic frequency						
AA	43	24.4%	67	38.1%	1.00 (Reference)	0.0222
AG	105	59.7%	86	48.9%	0.59 (0.36-0.94)	
GG	28	15.9%	23	13.0%	0.56 (0.29-1.10)	
Carrier comparison						
AA+AG	148	84.1%	153	86.9%	1.00 (Reference)	NS
GG	28	15.9%	23	13.1%	0.79 (0.44-1.44)	
AA	43	24.4%	67	38.1%	1.00 (Reference)	0.0080
AG+GG	133	75.6%	109	61.9%	0.42 (0.26-0.68)	

Allelic frequency

Allele A	191	54.3%	220	62.5%	1.00 (Reference)	0.0322
Allele G	161	45.7%	132	37.5%	0.71 (0.53-0.96)	

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 nasopharyngeal carcinoma patient and control groups.

C1722G (rs678653)	Controls		Patients		OR (95% CI)	<i>P</i> -value ^a
	n	%	n	%		
Genetic frequency						
GG	124	70.5%	127	72.2%	1.00 (Reference)	NS
CG	38	21.6%	37	21.0%	0.95 (0.57-1.59)	
CC	14	7.9%	12	6.8%	0.84 (0.37-1.88)	
Carrier comparison						
GG+CG	162	92.1%	164	93.2%	1.00 (Reference)	NS
CC	14	7.9%	12	6.8%	0.85 (0.38-1.89)	
GG	124	70.5%	127	72.2%	1.00 (Reference)	NS
CG+CC	52	29.5%	49	27.8%	0.92 (0.58-1.46)	

Allelic frequency

Allele G	286	81.3%	291	82.7%	1.00 (Reference)	NS
Allele C	66	18.7%	61	17.3%	0.91 (0.62-1.33)	

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

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

Variable	<i>CCND1</i> A870G (rs9344) genotype			<i>P</i> -value ^a
	AA (%)	AG (%)	GG (%)	
Smokers				
Controls	18 (24.7%)	44 (60.3%)	11 (15.0%)	0.0165^b
Patients	35 (45.5%)	37 (48.0%)	5 (6.5%)	
Non-smokers				
Controls	25 (24.3%)	61 (59.2%)	17 (16.5%)	NS
Patients	32 (31.1%)	49 (47.6%)	18 (17.4%)	

^a Based on Chi-square test, ^b Statistically significant, NS: non-significant.