Interaction of *CCND1* Genotype and Smoking Habit in Taiwan Lung Cancer Patients

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Abstract. *Aim: Cyclin D1 (CCND1) is critical in the transition of the cell cycle from G1 to S phase and unbalanced cell cycle regulation is a hallmark of carcinogenesis. The study aimed at investigating the association of CCND1 genotypes with lung cancer risk in Taiwan and examining the interaction between CCND1 genotype and smoking habit. Patients and Methods: CCND1 A870G (rs9344) and C1722G (rs678653) genotypes were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of DNA from the blood of 358 lung cancer patients and 716 cancer-free healthy controls. Results: The results showed that there were significant differences between lung cancer and control groups in the distribution of the genotypes (p=0.0003) and allelic frequency (p=0.0007) in the CCND1 rs9344 genotype. Individuals who carried AG or GG genotype had 0.59- and 0.52-fold risk, respectively, of developing lung cancer compared to those who carried the AA genotype (95% CI=0.44-0.78 and 0.35-0.79, respectively). There was also an obvious interaction of CCND1 rs9344 genotype with personal smoking habit on lung cancer risk (p=0.0009). Conclusion: These findings support the conclusion that cell cycle regulation may play a role in lung cancer development and that CCND1 rs9344 polymorphism together with smoking habit maybe a useful biomarker for lung cancer prediction.*

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Smoking may induce oxidative insults to the human genome, with the production of major DNA adducts of 8-hydroxy-2-deoxyguanine (8-OH-dG) (1, 2). 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 (1, 2). Thus, smoking habit is one of the main factors relevant to lung cancer. However, although tobacco smoking is the major risk factor in the development of lung cancer, only 10-15% of all smokers develop lung cancer, suggesting that there is a great variation among individuals in their susceptibility to lung carcinogenesis (3, 4). In Taiwan, lung cancer has high incidence, high mortality, and a low 5-year survival rate, especially in female adenocarcinoma cases (5).

Recent molecular biological studies have shown that lung cancer may be caused by the accumulation of multiple genetic defects including these of tumor suppressor genes, oncogenes, and DNA repair genes (6, 7). Cytogenetic studies have also shown that lung cancer cells may frequently contain chromosomal abnormalities as do other type of cancer cells (8). A number of surveillance mechanisms exist in cells to ensure the maintenance of genomic stability under various types of damage (9). Among them, the G_1/S checkpoint arrests the cell cycle to prevent replication of damaged DNA and allow DNA damage to be repaired (10, 11). Playing a role in the transition from G_1 to S phase of the cell cycle, cyclin D1 (CCND1) is considered as an essential regulator for this process, whose deregulation has been implicated in pathogenesis of several types of cancers, including lung cancer (12, 13). Some reports demonstrated that CCND1 may be involved in the development of some types of cancer in a cyclin-dependent kinase pattern (14, 15). Mechanisms of gene amplification, posttranscriptional or posttranslational modifications, rearrangements, and gene polymorphisms can result in abnormal protein levels and impaired CCND1 function, which can lead to carcinogenesis

Table I. *Characteristics of lung cancer patients and controls.*

Characteristic	Controls (n=716)			Patients (n=358)			*P*-value[a]
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			64.8 (6.8)			64.0 (6.9)	0.58
Gender							0.36
Male	488	68.1%		254	70.9%		
Female	228	31.9%		104	29.1%		
Habit							
Cigarette smokers	563	78.6%		293	81.8%		0.23
Non-smokers	153	21.4%		65	18.2%		

[a]Based on Chi-square test.

(16-18). However, the underlying mechanisms of CCND1 overexpression and its relationship to lung cancer are still not understood.

In the literature, there are some studies which investigated the association of *CCND1* genetic polymorphisms with lung cancer risk (19-21), and clinical outcomes (21, 22). In this study, we aimed at evaluating the contribution of *CCND1* polymorphisms to lung cancer in Taiwan, where non-small cell lung adenocarcinoma is prevalent. In addition, we also aimed at investigating the genotype interaction with smoking behavior.

Patients and Methods

Study population and sample collection. Three hundred and fifty-eight cancer patients diagnosed with lung cancer were recruited at the outpatient clinics of general surgery between 2005-2008 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. Twice as many lung cancer-free healthy volunteers as controls were selected by matching for age, gender and smoking habit 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 questions related to smoking habits. The 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 (23-28). 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): 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.

RFLP conditions. 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 the digestible G form. 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 that from the C allele was cut into fragments of 137 and 22 bp. Then, Product (10 μl) 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 controls 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 the 358 lung cancer and 716 control groups in their distributions of age, sex, and smoking status (Table I). In Table II, the frequencies of the genotypes and alleles of the *CCND1* A870G (rs9344) in the lung cancer and control groups are presented. The genotype of *CCND1* A870G was differentially distributed in control and case groups (p=0.0003). The odds ratios for the AG and GG were 0.59 (95% CI=0.44-0.78) and 0.52 (95%

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

A870G (rs9344)	Controls		Patients			
	n	%	n	%	OR (95% CI)	*P*-value[a]
Genetic frequency						
AA	175	24.4%	129	34.4%	1.00 (Reference)	**0.0003**
AG	422	58.9%	183	52.1%	**0.59 (0.44-0.78)**	
GG	119	16.6%	46	13.5%	**0.52 (0.35-0.79)**	
Carrier comparison						
AA+AG	597	83.4%	312	87.2%	1.00 (Reference)	0.1268
GG	119	16.6%	46	12.8%	0.74 (0.51-1.07)	
AA	175	24.4%	129	36.0%	1.00 (Reference)	**0.0001**
AG+GG	541	75.6%	229	64.0%	0.57 (0.44-0.76)	
Allelic frequency						
Allele A	772	53.9%	441	61.6%	1.00 (Reference)	**0.0007**
Allele G	660	46.1%	275	38.4%	**0.73 (0.61-0.88)**	

OR: Odds ratio, CI: confidence interval; [a]Based on Chi-square test, those values in bold are significant.

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

C1722G (rs678653)	Controls		Patients			
	n	%	n	%	OR (95% CI)	*P*-value[a]
Genetic frequency						
GG	514	71.8%	243	67.9%	1.00 (Reference)	0.3447
CG	152	21.2%	83	23.2%	1.16 (0.85-1.57)	
CC	50	7.0%	32	8.9%	1.35 (0.85-2.16)	
Carrier comparison						
GG+CG	666	93.0%	326	91.1%	1.00 (Reference)	0.2733
CC	50	7.0%	32	8.9%	1.31 (0.82-2.08)	
GG	514	71.8%	243	67.9%	1.00 (Reference)	0.2015
CG+CC	202	28.2%	115	32.1%	1.20 (0.91-1.59)	
Allelic frequency						
Allele G	1180	82.4%	569	79.5%	1.00 (Reference)	0.0994
Allele C	252	17.6%	147	20.5%	1.21 (0.96-1.52)	

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

CI=0.35-0.79), respectively, compared to the AA wild-type genotype. The differential distribution between the AG+GG *versus* AA genotype (OR=0.57, 95% CI=0.44-0.76), also suggested that people with either AG or GG genotype were at lower lung cancer risk. As for allelic frequency analysis, those who had a G allele were shown to have lower lung cancer risk (OR=0.73, 95% CI=0.61-0.88) compared with those had an A allele. On the contrary, for *CNND1* C1722G, there was no difference in the distributions of either genotype or allele between lung cancer patient and control groups (Table III). The conclusive finding deduced from the data in Tables II and III is that *CNND1* A870G may be a very potential biomarker of lung cancer, and the presence of G allele of *CNND1* A870G seems to be protective factor for lung cancer in Taiwan.

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

Variable	*CCND1* A870G (rs9344) genotype			
	AA (%)	AG (%)	GG (%)	*P*-value[a]
Smokers				
Controls	138 (24.5%)	332 (59.0%)	93 (16.5%)	**0.0009**
Patients	107 (36.5%)	150 (51.2%)	36 (12.3%)	
Non-smokers				
Controls	37 (24.1%)	90 (58.8%)	26 (17.0%)	0.3379
Patients	22 (33.8%)	33 (50.8%)	10 (15.4%)	

[a]Based on Chi-square test, statistical significant values are shown in bold.

A smoking habit is a lifestyle risk factor for lung cancer. The genotype distribution of the genetic polymorphisms of *CNND1* A870G was significantly different between lung cancer and control groups who have a smoking habit (p=0.0009) (Table IV). Consistent with the findings shown in Table II, the AG and GG genotype frequency was much lower (51.2% and 12.3%, respectively) in lung cancer patients who have smoking habit than in smoking controls (59.0% and 16.5%, respectively). There was no differential distribution in the non-smoking groups (p>0.05).

Discussion

To investigate the role of *CNND1* in lung cancer, we genotyped two most commonly studied polymorphic sites of the *CNND1* gene, A870G (rs9344) and C1722G (rs678653), and clarified their associations with the susceptibility for lung cancer risk in Taiwan. The data showed that the G variant genotypes of *CNND1* A870G were significantly associated with a lower susceptibility for lung cancer (Table II), and this genotype had joint effects with individual smoking habits on lung cancer susceptibility (Table IV), while the *CNND1* C1722G polymorphism dose not appear to play as an important role as *CNND1* A870G in lung carcinogenesis (Table III). As we hypothesized, the *CNND1* genotype indeed appears to play a role in determining lung cancer risk in Taiwan.

In the literature, several studies showed that the genotypes of *CNND1* A870G were associated with lung cancer risk (19-21), and clinical outcome (21, 22). However, which genotype plays the more critical role remains unclear and it is quite cancer type- and ethnic-dependent. Moreover, there has been no study performed for the Taiwanese population, which is world leading in the mortality of non-small cell lung adenocarcinoma in females. Consistent with our findings, the G allele seems to be a protective factor not only in lung cancer (19, 20), but also in hepatocellular carcinoma (29), laryngeal (30), breast (31), colorectal (32, 33), and bladder cancer (34). But controversially, the G allele was reported as risky for oral (35) and colorectal cancer (36), and not associated with lung (21) or other types of cancer (37-41).

To sum up, this study focused on the *CNND1* genotype and its joint effects with smoking habit on lung cancer risk in Taiwan. We found that the genotype of *CNND1* A870G, in association with smoking habit, may play an important role in lung cancer risk determination.

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