Interaction of CCND1 genotype and smoking habit in Taiwan lung cancer patients

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Running title: Hsia *et al*: *Cyclin D1* genotypes in lung cancer

Abstract. **Aim:** The *cyclin D1 (CCND1)* is critical in the transition of cell cycle from G1 to S phase and unbalanced cell cycle regulation is a hallmarker of carcinogenesis. The study aimed at investigating the association of *CCND1* genotypes with lung cancer risk in Taiwan and examining the gene-environment interaction among *CCND1* genotype and smoking habits. **Materials:** The genotype of *CCND1* A870G (rs9344) and C1722G (rs678653) were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of DNA from the blood. The study recruited 358 lung cancer patients and 716 cancer free health 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 of odds ratio 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 the 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.

Key Words: Cyclin D1, CCND1, polymorphism, lung cancer, smoking.

The smoking of tobacco is the most prevalent cause of lung cancer, which is the leading cause of cancer mortality in the world towards the end of the 20th century (1). Smoking may induce oxidative insults to the human genome, with the major DNA adducts of 8-hydroxy-2-deoxyguanine (8-OH-dG) (2, 3). 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 (2, 3). Thus, smoking habit is one of the main factors for 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 (4, 5). In Taiwan, lung cancer has high incidence, high mortality, and a low 5-year survival rate, especially in female adenocarcinoma cases (6).

Recent molecular biological studies have reported that lung cancer may be caused by the accumulation of multiple genetic defects including tumor suppressor genes, oncogenes, and DNA repair genes (7, 8). On the other way, cytogenetic studies have reported that lung cancer cells may frequently contain chromosomal abnormalities as other cancer cells (9). A number of surveillance mechanisms exit in cells to ensure the maintenance of genomic stability against various types of damage to our genome (10). Among them, the G1/S checkpoint arrests the cell cycle to

prevent replication of damaged DNA and allow DNA damage to be repaired (11, 12).

Playing a role in the transition from G1 to S phase of the cell cycle, cyclin D1 (CCND1) has been considered as a key regulator essential for this progress, whose deregulation has been implicated in pathogenesis of several cancers, including lung cancer (13, 14).

CCND1 is a critical protein at the G1-S phase checkpoint of the cell cycle (10, 11). Some reports demonstrated that CCND1 may involve in the development of some cancers in a CDK-independent pattern (15, 16). Mechanisms of gene amplification, posttranscriptional or posttranslational modifications, rearrangements, and gene polymorphisms can result in abnormal protein levels and impaired CCND1 function, which lead to carcinogenesis (17-19). However, the underlying mechanisms of the CCND1 overexpression and its relationship to lung cancer are still not understood. In the literature, there were a few papers investigated the association of *CCND1* genetic polymorphisms with lung cancer risk (20-22), and clinical outcomes (22, 23). In this study, we aimed at evaluating the contribution of *CCND1* polymorphisms to lung cancer in Taiwan, where the non-small cell lung adenocarcinoma is prevalent and causing a severe death. In addition, we also aimed at investigating the genotype joint interaction with smoking behaviors.

Materials 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 non-lung cancer healthy volunteers as controls were selected by matching for

age, gender and smoking 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 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 (24-29). 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°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. 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 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 were presented. The genotype of *CCND1* A870G was differentially distributed in control and case groups (*P*=0.0003). The odds ratios of the AG and GG were 0.59 (95%CI=0.44-0.78) and 0.52 (95%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 of either AG or GG genotypes were of lower lung cancer risk. As for allele

frequency analysis, those who had G allele were shown to have lower lung cancer risk

(OR=0.73, 95%CI=0.61-0.88), compared with those had A allele. On the contrary, as

for the *CNND1* C1722G, there was no difference in the distributions of either

genotype or allelic frequency between lung cancer patient and control groups (Table

III). The conclusive finding deduced from the data in Tables II and III is that the

CNND1 A870G can be a very potential biomarker, and the G allele of *CNND1* A870G

seems to be protective factor for lung cancer in Taiwan.

The smoking habit is an environmental risk factor for lung cancer. Therefore we

are interested in the interaction of genotype of *CNND1* A870G and the smoking

habits. The genotype distribution of various genetic polymorphisms of *CNND1*

A870G was significantly different between lung cancer and control groups who have

smoking habit (*P*=0.0009) (Table IV). Consistent with the findings in Table II, the

AG and GG genotype frequency was much lower (51.2% and 12.3%) in lung cancer

patients who have smoking habit than in smoking controls (59.0% and 16.5%). There

was no differential distribution in the non-smoking groups (*P*>0.05).

Discussion

To investigate the role of *CNND1* in lung cancer, we had genotyped 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 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

(Tables II), and this genotype had joint effects with individual smoking habits on lung

cancer susceptibility (Table IV), while the *CNND1* C1722G polymorphism may play

not as important role as *CNND1* A870G in lung carcinogenesis (Table III). As we

hypothesized, the *CNND1* genotype indeed played 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 risks (20-22), and clinical outcome (22, 23).

However which genotype plays more critical remains unclear and it is quite cancer

type- and ethic-dependent. Also, there was none study performed in Taiwan

population, which is world leading in the mortality of non-small cell lung

adenocarcinoma in female. Consistent with our findings, the G allele seems to be

protective factor not only in lung cancer (20, 21), but also in hepatocellular carcinoma

(30), larynx (31), breast (32), colorectal (33, 34), and bladder cancers (35). But

controversial findings reported that the G allele was risky for oral (36) and colorectal

cancer (37), or not associated in lung (22) or other cancers (38-42).

To sum up, this is a study which focuses 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, interacts with smoking habits, may play an important role in the lung cancer risk determination.

Acknowledgements

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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.

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

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

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

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

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

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

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

Variable	*CCND1* A870G (rs9344) genotype			
	AA (%)	AG (%)	GG (%)	*P*-value[a]
Smokers				
Controls	138 (24.5%)	332 (59.0%)	93 (16.5%)	**0.0009[b]**
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; [b] Statistical significant based on Chi-square test.