The Significant Association of *CCND1* Genotypes with Gastric Cancer in Taiwan

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Abstract. *Background and Aim: Gastric cancer is one of the most common malignant tumors worldwide. Due to the complex initiation and intricate progression mechanisms, early detection and effective treatment of gastric cancer are both difficult to achieve. The genetic polymorphisms encoding critical protein cyclin D1 (CCND1) to regulate cell cycle transition from G1 phase to S phase may determine the susceptibility of individuals to gastric cancer. The study aimed to examine the contribution of CCND1 genotypes to gastric cancer risk in Taiwan. Materials and Methods: The genotypes of CCND1 A870G (rs9344) and G1722C (rs678653) were determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis among 358 gastric patients and 358 cancer-free controls, and the distribution of genotypic and allelic frequencies among the two groups were compared. Results: The results showed that there were significant differences between gastric cancer and control groups in the distribution of the genotypes ($p=6.86\times10^{-4}$) and allelic frequency (p=0.0016) in the CCND1 A870G genotype. In addition, individuals who carried the AG or GG genotype had 0.55- and 0.51-fold of odds ratios of developing gastric cancer compared to those who carried the AA genotype (95% confidence intervals [CI]=0.39-0.76 and 0.32-0.81, respectively). There was no such association of CCND1 G1722C with gastric cancer. Furthermore, there was an obvious interaction of the CCND1 A870G genotype with personal smoking habit on gastric cancer risk (p=0.0005). Conclusion: Cell-cycle regulation may play a role in gastric cancer initiation and development and the CCND1 A870G genotype maybe a useful biomarker for detection of early gastric cancer.*

Gastric cancer is reported to be more common in males and elder citizens, aged 50 years or older (1). Even during these decades several beneficial developments, such as the increasing use of refrigerators, the lowering dependence on salts to preserve food, the elevating availability and intake of fresh fruits and vegetables and the effective control of chronic infection with *H. pylori*, decreased the incidence of gastric cancer, however, it remained as a critical cancer accounting for 8% of the total cancer incidence and 10% of the total cancer death worldwide (2). Clinically, the prognosis of gastric cancer is usually poor with a 5-year survival less than 20% for advanced disease (3) because most tumors are currently diagnosed at advanced stages of the disease. The detection of gastric cancer at earlier stages probably gives the best chance to improve prognosis and the determination of individuals with high cancer risk provides us a promising approach to achieve this goal.

The *cyclin D1* (*CCND1*) gene located on human chromosome 1q31-32 is expressed to regulate the transition through the restriction point in the G_1 phase to S phase of the

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Table I. *Distributions of characteristics of gastric cancer patients and healthy controls.*

Characteristics	Controls (n=358)		Cancer patients (n=358)		*p*-Value[a]
	n	%	n	%	
Mean Age (SD)	62.1 (9.5)		63.8 (11.4)		0.5811
Gender					0.2219
Male	242	67.6%	258	72.1%	
Female	116	32.4%	100	27.9%	
Histologic subtype					
Intestinal type			201	56.2%	
Diffuse type			157	43.8%	
Stage					
Early stage			173	48.3%	
Advanced stage			185	51.7%	
Personal smoking status					
Cigarette smokers	234	65.4%	256	71.5%	0.0912
Non-smokers	124	34.6%	102	28.5%	

[a]*p*-Value based on two-sided Chi-square test without Yate's correction.

cell cycle. The mechanisms of *CCND1* gene amplification, post-transcriptional or post-translational modifications, rearrangements and polymorphisms can result in abnormal protein levels and impaired CCND1 function, which may be closely-related to carcinogenesis (4). In other types of cancers, such as cutaneous melanoma, esophageal squamous cell carcinoma, breast cancer and bladder cancer, high expression of CCND1 was frequently found in the tumor cites (5-8). Besides gene amplification, the transcriptional mRNA and translational protein levels of CCND1 may be controlled by *CCND1* genetic polymorphism (9). For instance, the A870G single nucleotide polymorphism (SNP) (rs9344) was associated with an increased risk for cancer development or poor prognosis in squamous cell carcinoma of the head and neck (10, 11), pituitary adenoma (12), lung cancer (13) and urothelial carcinoma (14).

In the literature, the genotypes at *CCND1* A870G (rs9344) were reported to be associated with gastric cancer in Japanese (15, 16), Korean (17) and Chinese populations (18). However, whether other SNP at *CCND1* may contribute to gastric cancer or *CCND1* may contribute to Taiwan gastric cancer susceptibility has been never studied. Thus, the specific aim of this study was to determine the genotypic frequency of two SNPs of *CCND1*, A870G (rs9344) and G1722C (rs678653), in Taiwan gastric cancer population and the possibility to serve as potential biomarkers for the detection of early gastric cancer.

Materials and Methods

Collection of investigated populations. Three hundred and fifty eight patients diagnosed by experienced surgeons with gastric cancer were included at the out-patient clinics of general surgery at China Medical University Hospital during 2001 to 2009. This study also comprised the same number of non-cancer healthy people selected by matching for age and gender as controls 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. All subjects who voluntarily participated, completed a self-administered questionnaire and provided their peripheral blood samples for DNA extraction and genotyping. The details of the characteristics of the patients and controls were summarized in Table I.

CCND1 genotyping conditions. Genomic DNA of each participant was extracted from peripheral blood leucocytes, aliquoted and processed according as previously described (10, 11, 13). 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* G1722C (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; with a final extension at 72˚C for 10 min.

Restriction fragment length polymorphism (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. In the results of gel electrophoresis, two fragments of 145 and 22 bps were presented for the genotype G form while one 167-bp fragment was presented for the genotype A 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 the 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

Table II. *Distribution of CCND1 A870G genetic and allelic frequencies among gastric cancer patients and controls.*

A870G (rs9344)	Controls	%	Cases	%	OR (95% CI)[a]	*p*-Value[b]
Genetic frequency						
AA	87	24.3%	134	37.4%	1.00 (Reference)	**$6.86*10^{-4}$**
AG	212	59.2%	178	49.7%	**0.55 (0.39-0.76)**	
GG	59	16.5%	46	12.9%	**0.51 (0.32-0.81)**	
Carrier comparison						
AA+AG	299	83.5%	312	87.1%	1.00 (Reference)	0.2047
GG	59	16.5%	46	12.9%	0.75 (0.49-1.13)	
AA	87	24.3%	134	37.4%	1.00 (Reference)	**0.0002**
AG+GG	271	75.7%	224	62.6%	**0.54 (0.39-0.74)**	
Allele frequency						
Allele A	386	53.9%	446	62.3%	1.00 (Reference)	**0.0016**
Allele G	330	46.1%	270	37.7%	**0.71 (0.57-0.87)**	

[a]OR, Odds ratio; CI, confidence interval; [b]Based on Chi-square test, values in bold were statistically significant.

Table III. *Distribution of CCND1 G1722C (rs678653) genetic and allelic frequencies among gastric cancer patients and controls.*

G1722C (rs678653)	Controls	%	Cases	%	OR (95% CI)[a]	*p*-Value[b]
Genetic frequency						
GG	252	70.4%	245	68.4%	1.00 (Reference)	0.8509
CG	78	21.8%	83	23.2%	1.09 (0.77-1.56)	
CC	28	7.8%	30	8.4%	1.10 (0.64-1.90)	
Carrier comparison						
GG+CG	330	92.2%	328	91.6%	1.00 (Reference)	0.8912
CC	28	7.8%	30	8.4%	1.08 (0.63-1.84)	
GG	252	70.4%	245	68.4%	1.00 (Reference)	0.6266
CG+CC	106	29.6%	113	31.6%	1.10 (0.80-1.51)	
Allele frequency						
Allele G	582	81.3%	573	80.0%	1.00 (Reference)	0.5926
Allele C	134	18.7%	143	20.0%	1.08 (0.83-1.41)	

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

electrophoresis. The genotype analysis was performed by two researchers independently and blindly and the results were 100% concordant.

Statistical analyses. The 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. The associations between the *CCND1* polymorphisms and gastric cancer risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from unconditional logistic regression analysis with the adjustment for possible confounders. $P<0.05$ was considered statistically significant and all statistical tests were two-sided.

Results

The characteristics of age, gender and cigarette smoking habits of all the investigated subjects together with the histological subtype and cancer stage of the gastric cancer patients are summarized in Table I. The data showed that there was no difference in the distribution of age ($p=0.5811$), gender ($p=0.2219$) and personal cigarette smoking status ($p=0.0912$) among gastric cancer patients and non-cancer controls (Table I).

In Table II, the frequencies of the genotypes and alleles of the *CCND1* A870G among the investigated gastric cancer patients and non-cancer healthy controls were presented and analyzed. The results showed that the genotypic frequencies of *CCND1* A870G was differentially distributed between the control and cancer patient groups ($p=6.86\times10^{-4}$). The odds ratios of the hetero- and homozygous variant AG and GG were 0.55 (95%CI=0.39-0.76) and 0.51 (95%CI=0.32-0.81), respectively, compared with the wild-type AA genotype. The comparison model of the AG+GG *versus* AA genotype (OR=0.54, 95%CI=0.39-0.74) also suggested that people of either AG or GG genotypes were of lower gastric cancer risk

Table IV. *Distribution of CCND1 A870G (rs9344) genotypes in gastric cancer patients after stratification by personal cigarrete smoking status.*

Genotypes	Non-smokers		*p*-Value	OR (95% CI)[a]	Smokers		*p*-Value	OR (95%CI)[a]
	Controls (%)	Cases (%)			Controls (%)	Cases (%)		
AA	30 (26.3)	34 (33.3)	0.1401	1.000 (Reference)	57 (24.4)	100 (39.1)	0.0005*	1.000 (Reference)
AG+GG	94 (73.7)	68 (66.7)		0.64 (0.36-1.14)	177 (75.6)	156 (60.9)		0.50 (0.34-0.74)*
Total	124 (100)	102 (100)			234 (100)	256 (100)		

[a]OR, Odds ratio; CI: confidence interval; ORs were estimated with multivariate logistic regression analysis. *Statistically significant.

Table V. *Association of CCND1 A870G (rs9344) genotypes with gastric cancer risk among Asian populations.*

First author	Year	Population	Controls (n)	Cases (n)	Association
Kuo WH*	2014	Taiwanese	358	358 gastric cancer cases	Allele A associated with higher risk, especially in smokers
Tahara T	2010	Japanese		139 gastric cancer cases	G allele carriers had higher hypermethylation status at tumor suppressor genes, especially those at advanced stage
Tahara T	2009	Japanese	359	392 gastric cancer cases	No association
Tahara T	2008	Japanese	359	165 premalignant cases	Allele A associated with higher risk, especially in older people
Jia A	2008	Chinese	162	159 gastric cancer cases	Allele G associated with higher risk, especially in older people
Song JH	2007	Korean	442	253 gastric cancer cases	No association

*The article is the present study.

(Table II). As for the allelic frequency analysis, those who had the G allele were of lower gastric cancer risk (OR=0.71, 95%CI=0.57-0.87) than those who had the A allele at *CCND1* A870G. On the contrary, there was no difference in the distributions of either genotypic or allelic frequency between gastric cancer patient and control groups in relation to *CNND1* G1722C (Table III). The conclusive findings deduced from the data in Tables II and III is that *CNND1* A870G, but not *CNND1* G1722C, can be a potential biomarker for the detection of early gastric cancer. Also, the G allele at *CNND1* A870G seems to be a protective genetic factor for gastric cancer progression among the Taiwanese population.

Since smoking status is reported to be one of the environmental risk factors for gastric cancer in the literature, we were interested to investigate the interaction of the *CNND1* A870G genotype and personal smoking status for gastric cancer risk. As shown in Table IV, the genotypic distributions of *CCND1* A870G AA and AG+GG were significantly different between gastric cancer patient smokers and control smokers (p=0.0005) (Table IV). The AG+GG genotypic frequency was much lower (60.9%) in gastric cancer smokers than healthy smokers (75.6%) and patiets were at a lower gastric cancer risk (OR=0.50, 95%CI=0.34-0.74). There was no such differential distribution among the non-smokers (p>0.05) (Table IV).

Discussion

Previously, several potential genetic markers for the detection and prediction of early gastric cancer in Taiwan were proposed, including *caveolin-1* G14713A, *Ku70* T-991C, *exonuclease I* K589E and *XRCC4* G-1394T (19-22). Most of them are SNPs on the DNA repair system genes, while little is known about the contribution of personal genotypes of the cell-cycle regulation genes to gastric cancer risk. In the present study, we aimed at investigating the association of *CCND1* genotypes and gastric cancer risk in Taiwan. We have selected and genotyped two most commonly investigated polymorphic sites, *CCND1* A870G and G1722C, among the 358 gastric cancer cases and 358 age- and gender-matched controls. In Table II, it is shown that subjects carrying the AG and GG genotypes were of lower risk for gastric cancer compared with those carrying the AA genotype on *CCND1* A870G (Table II). The combination of AG and GG *versus* the AA wild-type is also significantly of lower risk for gastric cancer. In addition, the results from the allelic frequency analysis suggest that the G allele is a protective genomic marker for gastric cancer in Taiwan (Table II). As for *CCND1* G1722C, there was no similar differential genotypic or allelic distribution found (Table III). Furthermore, we found that there was a synergistic genetic-lifestyle interaction for *CCND1* A870G and personal

smoking status (Table IV). However, whether the *CCND1* A870G genotype has an interaction with other factors, such as *H. pylori* infection and/or fruit and vegetable intake, needs to be further investigated.

In the literature, the contribution of *CCND1* A870G genotypes to gastric cancer risk has been investigated by several Asian groups with conflicting findings that are summarized in Table V. In 2007, Song and his colleagues, in a study of 253 gastric cancer patients together with 442 cancer-free controls, found that there was no association between *CCND1* A870G genotypes and gastric cancer risk. They proposed that the male gastric cancer patients had a significantly higher proportion of the homozygous G/G genotype and that the *CCND1* A870G genotype could have contributed to observed gastric cancer risk in men (17). In 2008, a study in Chinese population demonstrated that the risk of gastric cancer for subjects with *CCND1* A870G GG or GA genotypes were 2.8- or 1.4-fold higher than those with AA genotype. Furthermore, in the stratification analyses, the risk of GG genotype was more evident in subjects equal to or older than 60 years of age and those positive for *H. pylori* infection (18). The findings were valuable but discounted by the limited sample size (159 cases and 162 controls), especially those collected from stratified analysis, demanding validation with larger samples in the near future. Tahara and his colleagues' continuous investigation added valuable information about the contribution of the *CCND1* A870G genotype in relation to gastric cancer risk. First, they recruited people with precancerous conditions, including 111 gastric and 54 duodenal ulcers, as well as 359 non-ulcer subjects, and found that AA genotype carriers held a significant high risk of intestinal metaplasia, especially in older subjects of 61 years or older (23). However, after comparing the *CCND1* A870G genotype of the 359 non-ulcer subjects with an equal amount of gastric cancer patients, no positive association was found (15). They further investigated the effects of the *CCND1* A870G genotype on the methylation status of the promoter regions of tumor suppressor genes, providing the first evidence that the *CCND1* A870G genotype might be involved in methylation-related gastric carcinogenesis, especially in the advanced stage (16). Even the Asian populations were of much more similar genetic background compared with the Western ones, the findings within the Asian countries are remaining conflicting and inclusive. In addition to genetic background, several factors should be taken into consideration, including the different criteria in the inclusion and exclusion of subjects, study grouping, recording of patient age at diagnosis, genotyping methodologies and most of all the lifestyle background.

To sum up, the current study reports that *CCND1* A870G genotypes, synergistically interacted with personal cigarette smoking habit and may increase the personal risk to gastric carcinogenesis in Taiwan. The results provide evidence supporting that gastric carcinogenesis is a complex pathway that involves both inherited and environmental factors. The AG and GG genotypes of *CCND1* A870G may be a protective marker in gastric oncology for early detection and prediction.

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