Association of *cyclin D1* G870A polymorphism with susceptibility to gastric cancers in Korean male patients

J. H. SONG[1], C. J. KIM[1], Y. G. CHO[1], Y. K. PARK[2], S. W. NAM[1], N. J. YOO[1], J. Y. LEE[1], W. S. PARK[1*]

[1]Department of Pathology, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea, e-mail: wonsang@catholic.ac.kr; [2]Department of Biostatistics, College of Medicine, The Catholic University of Korea, 505 Banpo-dong, Seocho-gu, Seoul 137-701, Korea

Received November 14, 2006

Cyclin D1 is a key cell cycle regulator that is upregulated in gastric cancer. The common G870A polymorphism of *cyclin D1* which can influence cancer susceptibility and disease outcome has been the most frequently investigated. The specific aim of this study is to investigate whether the G870A polymorphism of *cyclin D1* was associated with individual susceptibility to gastric cancer in Korea. The frequency of the polymorphism was examined in 253 gastric cancer patients and 442 healthy controls. Polymorphism analysis was performed by amplifying exon 4 of *cyclin D1* and sequencing the products. The frequencies of genotypes: G/G, G/A and A/A were 28.1% (71/253), 49.4% (125/253) and 22.5% (57/253), respectively, in gastric cancer cases, and 23.1%, 51.1% and 25.8%, respectively, in healthy controls. Statistically, the polymorphism was not associated with increased risk of gastric cancer. When stratified by histological subtype of gastric cancer, the risk was also not statistically significant. However, the male gastric cancer patients showed a significantly higher proportion of the homozygous G/G genotype and the G allele (Chi-Square test, $P = 0.0242$ & $P = 0.0307$) compared to males in the control group. Thus, our findings suggested that the G870A polymorphism of *cyclin D1* was not associated with an increased risk for gastric cancer in this population; however, it may contribute to susceptibility to gastric cancer in men.

Key words: *Cyclin D1, Gastric cancer, Polymorphism, Susceptibility*

Gastric cancer has a high incidence in Asia and is one of the most common causes of cancer death in the world. In Korea, it accounts for an estimated 20.2% of all malignancies, 24.0% in males and 15.3% in females [1]. However, little is known about the molecular genetic events involved in the development and progression of gastric cancer.

Wnt/β-catenin signaling plays an essential role in both development and tumorigenesis [2, 3]. Genetic alterations of the Wnt/β-catenin signaling pathway related genes promote cell proliferation and inhibit differentiation, ultimately leading to tumor formation [4]. Increased activity of the Wnt/β-catenin signaling pathway has been frequently detected in gastric cancers [5]. Some of the targeted genes of the APC–*β*-catenin–Tcf pathway have been identified in vitro, and cyclin D1 is one of them [6, 7]. Cyclin D1 is a key cell cycle regulator that is upregulated in gastric cancer [8, 9].

Over 100 single nucleotide polymorphisms (SNPs) have been identified spanning the *cyclin D1* locus; the common G870A SNP has been the most frequently investigated. The polymorphism that modulates mRNA splicing to produce two transcripts: the full-length transcript in the "G" allele and a shorter transcript terminating at intron 4 in the "A" allele [10]. Although both transcripts encode proteins that contain the functional cyclin box (amino acid 55-161), the shorter transcript does not contain the exon 5 sequence encoding a protein destabilizing destruction box responsible for the rapid turnover of the protein. Because of the significance of cyclin D1 in human cancer, a large number of epidemiological studies have evaluated the influence of this particular polymorphism in cancer susceptibility and disease outcome. Previous studies have shown that the G870A polymorphism of *cyclin D1* influences susceptibility to colorectal cancer, breast cancer, parathyroid adenoma, von Hippel-Lindau disease and squa-

This study was supported by a grant of the Korea Health 21 R&D Project (A060093), Ministry of Health & Welfare, Republic of Korea.

* Corresponding author

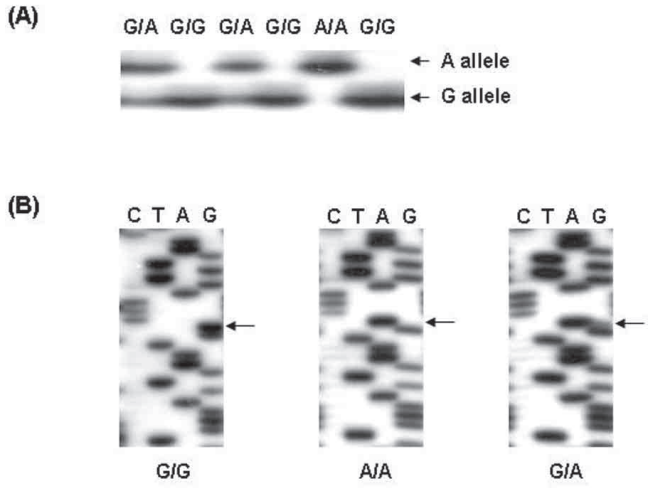

Figure 1. Genotypes analysis at G870A polymorphism site of the *cyclin D1* gene: SSCP band patterns (A) and sequencing (B) of homozygote G/G and A/A, and heterozygote with G/A (arrow).

mous cell carcinoma of the head and neck [11–16]. Although the majority of studies link the A-allele, especially the A/A genotype, to an increased cancer risk and poor prognosis, some studies have implicated the G-allele in increased cancer risk or found no association at all. For gastric cancer, it has been reported that the polymorphism is associated with increased risk of cancer for patients in the United States [17] but not in Germany [18].

In the present study, we investigated whether the G870A polymorphism of *cyclin D1* was associated with individual susceptibility to gastric cancer in Korea. In addition, we studied whether the polymorphism was associated with the histological classification of intestinal- and diffuse-type gastric cancer.

Materials and methods

Tissue samples. Archival normal gastric mucosa specimens from 253 gastric cancer patients who had undergone surgery at the College of Medicine, The Catholic University of Korea in Seoul, between 2000 and 2003 were included in this study. All neoplasms were pathologically confirmed as stomach adenocarcinomas. The 253 cases included 165 men (65.2%) and 88 women (34.8%) with a median age of 61 (22–85) years at initial diagnosis. Histologically, the cancers consisted of 134 intestinal-type (53.0%) and 119 diffuse-type (47.0%) gastric cancers. The healthy control population consisted of 208 males and 234 females with a mean age of 45. To exclude ethnic differences, only a Korean population was included in this study. Informed consents were obtained according to the Declaration of Helsinki. This study was approved by the institutional review board of the Catholic University of Korea, College of Medicine.

DNA extraction. Normal cells were obtained from the cancer-free gastric mucosa. The DNA extraction was performed by a modified single-step DNA extraction method, as was described previously [19]. For the control population, a leukocyte cell pellet from each blood sample was obtained from the buffy coat by centrifugation of 2 ml of whole blood. The cell pellet was used for DNA extraction. The Qiagen DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) was used according to the manufacturer's instructions to obtain genomic DNA. The DNA purity and concentration were determined by Nanodrop® ND-1000 spectrophotometer (Nanodrop technologies, Wilmington, DE, USA).

SSCP and DNA sequencing. Genomic DNAs from gastric mucosal tissues and blood samples were amplified with primers covering the *cyclin D1* polymorphism in exon 4. For PCR, the primer sequences were as follow: Sense 5'-tactaccgcc tcacacgcttcc-3' and antisense 5'-ttggcaccagcctcggcatttc-3'. Each polymerase chain reaction (PCR) procedure was performed under standard conditions in a 10 μl reaction mixture containing 1 μl of template DNA, 0.5 μM of each primer, 0.2 μM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 0.4 unit of Ampli Taq gold polymerase (Perkin-Elmer, Foster City, CA, USA), 0.5 μCi of [^{32}P]dCTP (Amersham, Buckinghamshire, UK), and 1 μl of 10X buffer. The reaction mixture was denatured for 12 min at 94°C and then incubated for 35 cycles (denaturing for 30 s at 94°C, annealing for 30 s at 61°C and extension for 30 s at 72°C). A final extension step was performed for 5 min at 72°C. After amplification, the PCR products were denatured for 5 min at 95°C in a 1:1 dilution of sample buffer containing 98% formamide/5 mmol/L NaOH, and these products were loaded onto a SSCP gel (FMC Mutation Detection Enhancement system; Intermountain Scientific, Kaysville, UT, USA) with 10% glycerol. After electrophoresis, the gels were transferred to 3 MM Whatman paper and dried, and autoradiography was then performed using Kodak X-OMAT film (Eastman Kodak, Rochester, NY, USA). Sequencing of the PCR products was carried out using the cyclic sequencing kit (Perkin-Elmer, Foster City, CA, USA) according to the manufacturer's recommendation (Fig. 1).

Statistical analysis. The chi-square test for association was used to test differences of genotype frequencies between normal controls and gastric cancer patients, and between the two histological types. The genotype specific risks were estimated as odds ratios and 95% confidence intervals (CIs). The odd ratios and their 95% CIs were calculated by logistic regression analysis, with adjustment for age (in years) and sex.

Results

The genotype frequencies of G870A single nucleotide polymorphisms of *cyclin D1* in Korean gastric cancer cases and controls are summarized in Table 1. The frequencies of genotype G/G, G/A and A/A in normal healthy individuals were: 23.1%, 51.1% and 25.8%, respectively. The frequencies of A and G alleles were 51.4% and 48.6% in healthy control in-

Table 1. Distribution of *CCND1* genontype and allele frequency in gastric cancer patients and controls.

CCND1 genotype	Cases (n=253)		Controls (n=442)		Crude OR (95% CI)	Adjusted[a] OR (95% CI)
	Number	Percent	Number	Percent		
GG	71	28.1	102	23.1	1.00	1.00
GA	125	49.4	226	51.1	0.80 (0.55-1.15)	0.91 (0.58-1.43)
AA	57	22.5	114	25.8	0.72 (0.46-1.11)	0.75 (0.44-1.27)
G allele frequency[b]	0.528		0.486			
Trend test[c]					$P = 0.1374$	$P = 0.2836$

[a]Adjusted for age (in years) and sex

[b]Two-sided χ^2-test: for genotype distribution, $P = 0.3015$.

[c]Calculated in the logistic regression models using the number of G alleles in the genotypes as a continuous variable

Table 2. Stratification analysis of *CCND1* genotype frequencies in gastric cancer.

Variable	*CCND1* genotype						Adjusted OR[a] (95% CI)	
	No. of cases			No. of controls			GA versus GG	AA versus GG
	GG	GA	AA	GG	GA	AA		
Age (in years)								
≤ 50	12	27	12	77	167	86	1.01 (0.48-2.11)	0.87 (0.37-2.06)
> 50	59	98	45	25	59	28	0.77 (0.43-1.37)	0.69 (0.35-1.36)
Sex								
Male	51	75	39	39	112	57	1.10 (0.55-2.18)	0.98 (0.44-2.22)
Female	20	50	18	63	114	57	0.82 (0.45-1.52)	0.61 (0.30-1.25)
Lauren's								
Diffuse	31	63	25	$P = 0.5703$ (χ^2-test)				
Intestinal	40	62	32					

[a]Adjusted for the other covariates [age (in years) as a continuous variable] presented in this table in a logistic regression model for each stratum.

dividuals, respectively. Genotype frequencies of all groups were in accordance with those previously reported in the Caucasian population [20]. There was no statistical difference in the genotype and G/A allele frequencies in comparisons between males and females in healthy controls (Chi-Square test, $P = 0.1255$ & $P = 0.0958$).

For the gastric cancer patients, the G/G, G/A, A/A genotypes had a prevalence of: 28.1% (71/253), 49.4% (125/253) and 22.5% (57/253), respectively, and the frequencies of A and G alleles were 47.2% and 52.8%. Finally, we did not observe a significant difference in the genotypes and allele frequencies between the gastric cancer patients and healthy controls (Trend test, $P = 0.1374$, Chi-Square test, $P = 0.3015$), and between males and females with the gastric cancer (Chi-Square test, $P = 0.2094$ & $P = 0.5915$). Compared with the GG genotype, the adjusted OR for the GA and AA genotypes were 0.91 (95% CI 0.58-1.43) and 0.75 (95% CI 0.44-1.27), respectively. The trend test using a logistic regression model showed that the effects of genotype on the OR were not significant ($P = 0.2836$) after adjustment for age and sex. In the male population, gastric cancer patients showed a significant higher proportion of the homozygous G/G genotype and the G allele (Chi-Square test, $P = 0.0242$ & $P = 0.0307$) compared to the control group (Table 2). This finding suggested that the G870A polymorphism of *cyclin D1* may be closely associated with increased risk of gastric cancer in the male population. However, elevated risk for gastric cancer was not statistically significant in the female patients studied. In addition, when stratified by histological subtype of gastric cancer, the risk was not statistically significant (Chi-Square test, $P = 0.5703$) (Table 2).

Discussion

Many common diseases in humans, especially cancer, are not caused by one genetic variation within a single gene, but are determined by complex interactions among multiple genes, as well as environmental and lifestyle factors. Genetic factors, including polymorphisms of genes involved in tumorigenesis, may partly explain the difference in individual susceptibility to cancer [21].

CCND1 encodes cyclin D1, a key cell regulatory protein signalling the transition from G1 to the S phase of the cell cycle. Cyclin D1 overexpression was reported to be frequently seen in gastric cancer and associated with its poor differentiation [22]. The *cyclin D1* gene is composed of five distinct exons; the G870A polymorphism is found at the intron 4/exon 5 boundary. Interestingly, the G870 allele creates an optimal splice donor site, resulting in the full-length transcript *A*. By contrast, the A870 allele is predicted to hinder the splicing event, thus giv-

ing rise to a variant splice transcript *B* terminating at intron 4 [23]. Some studies have reported that cyclin D1b is constitutively nuclear in localization and that cyclin D1b may be more oncogenic in human cancer [24, 25]. Unexpectedly, individuals with the A/A genotype can still produce transcript A, thus indicating that the A870 allele is not completely penetrant for transcript b production, and individuals with the G/G genotype can produce transcript b [23]. Further investigation is needed to identify the relationship of the polymorphism to transcript b production and the factors which influence transcript a and b splicing independently of the polymorphic nucleotide.

In the present study, we found that the polymorphism studied may not contribute to individual risk for the development of gastric cancer in this population. However, in the stratification analysis, the G/G genotype and the G allele of the *cyclin D1* gene were closely associated with an increased risk of gastric cancer in men. According to the histological types, there were no significant differences in the genotype and allele frequencies of the polymorphism. Thus, we concluded that the G870A polymorphism of *cyclin D1* may be closely associated with increased risk of gastric cancer in the male population.

The different genetic background which varies from one geographic region or population to another may be very important in susceptibility to cancer. For the *cyclin D1* gene, the polymorphism frequency in the Korean population is approximately 48.6% for A and 51.4% for G; the frequencies of each allele were similar to those reported in the Caucasian population [20]. A majority of studies link the A870 allele to increased cancer risk and poor disease outcome, with the largest associations observed with the A/A genotype [26, 27]. Conversely, Hong *et al* has found that the GG genotype is associated with increased susceptibility and advanced colorectal cancer in patients from Singapore [28]. In German Caucasian population, the polymorphism was not associated with the risk for gastric cancer [18]. We also found a significant negative association between gastric cancer and the G870A polymorphism. However, when patients were stratified by gender, a significant difference in genotype and allele frequency of the G870A polymorphism was found in the male gastric cancer patients (Table 2). Although the background for explaining why the G allele is related to increased risk of gastric cancer in males remain unknown, since it has been suggested that *Helicobactor pylori* (*H. pylori)* infection is associated with increased cyclin D1 expression and gastric carcinogenesis [29], we cannot completely rule out the possibility that *H. pylori* may activate the cyclin D1 containing G allele. Thus, it is necessary to investigate whether the activation of cyclin D1, induced by *H. pylori,* is associated with the polymorphism. In addition, further studies in a large population with other risk factors including alcohol abuse and smoking, should be performed to confirm this initial observation.

Molecular, histological and epidemiological studies have provided evidence that gastric adenocarcinoma is a heterogeneous disease with two main histological types: the intestinal and the diffuse type [30]. Although both types seem to start from *H. pylori*-related chronic gastritis [31], epidemiologic and histopathological evidence have shown that gastric carcinoma may be influenced by genetic polymorphisms, age of cancer onset and gender. Unexpectedly, our study confirmed absence of a correlation between the *cyclin D1* G870A polymorphism and gastric cancer when the patients were stratified by histology.

In conclusion, the present study demonstrated that gastric cancer does not appear to be associated with the *cyclin D1* G870A polymorphism in Korean population. However, when the gastric cancer patients were stratified by gender, the G/G genotype and the G allele were associated with increased risk of gastric cancer in men. In addition, we found no association of gastric cancer with the polymorphism studied when patients were sorted by histological type. Further molecular genetic studies should be performed in a large population to identify the mechanisms associated with cyclin D1 activation by *H. pylori* infection.

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