KLF6 IVS1 -27G/A polymorphism with susceptibility to gastric cancers in Korean

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KLF6 is a key cell cycle regulator that is downregulated in several kinds of human cancers, including gastric cancer. The IVS1 -27G/A polymorphism of *KLF6* has been investigated, which can influence susceptibility to gastric cancer and disease outcome. In order to investigate whether the IVS1 -27G/A polymorphism of *KLF6* is associated with individual susceptibility to gastric cancer in Korea, the frequency of the polymorphism was examined in 264 gastric cancer patients and 299 healthy controls. Single nucleotide polymorphism (SNP) analysis was performed by amplifying intron 1 of *KLF6* and sequencing the products. The frequencies of genotypes: G/G, G/A and A/A were 91.7% (242/264), 5.7% (15/264) and 2.6%, respectively, in gastric cancer cases and 91.9%, 7.0% and 1.1%, respectively, in healthy controls. Genotype frequencies in Korean population were very similar to those of Caucasian population. Interestingly, the male gastric cancer patients showed a significantly higher proportion of the G allele (Chi-Square test, P=0.005) compared to female gastric cancer patients. However, the polymorphism was statistically not associated with increased risk of gastric cancer in Korea. When stratified by histological subtype of gastric cancer, the risk was also not statistically significant. Thus, our results suggested that the IVS1 -27G/A polymorphism of *KLF6* is not associated with an increased risk for gastric cancer in Korean population.

Key words: KLF6, Gastric cancer, Polymorphism, Susceptibility, Genotype,

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

Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor that is part of a growing KLF family [2, 3]. KLF6 is a tumor suppressor gene inactivated by allelic loss and somatic mutation in several human cancers, including hepatocellular carcinoma and prostate, gastric and colon cancers [4–8]. Recently, a germ line DNA single nucleotide polymorphism (SNP) have been identified in the intron 1 of the *KLF6* gene and is associated with increased prostate cancer risk [9]. This IVS1 -27G/A polymorphism generates a novel functional SRp40 DNA binding site and increases transcription of three alternatively spliced KLF6 isoform, which antagonize tumor suppressor function of wild type KLF6 [9]. Because of the significance of *KLF6* gene in human cancer, epidemiological studies have evaluated the influence of this particular polymorphism in cancer susceptibility and disease outcome. In previous studies, it has been found that the IVS1 -27G/A polymorphism of *KLF6* influences susceptibility to prostatic cancer and lung cancer [9, 10]. However, association of *KLF6* IVS1 -27G/A germline polymorphism with increased risk of prostatic cancer was not found in Finnish and Ashkenazi Jewish populations [11, 12].

In functional analysis, wild-type KLF6 up-regulates the cell cycle inhibitor p21 in a TP53-independent manner and it suppresses growth, whereas tumor-derived *KLF6* mutants fail to upregulate p21 or suppress cell proliferation in the prostatic and non-small cell lung cancer cells [8, 13]. In addition, KLF6 inhibits key oncogenic signaling pathways, such as the cyclin-dependent kinase complex CDK4-cyclin D1 [14] and E-cadherin is a novel transcriptional target of the KLF6 [15]. In our previous report, somatic mutations of the *KLF6* gene

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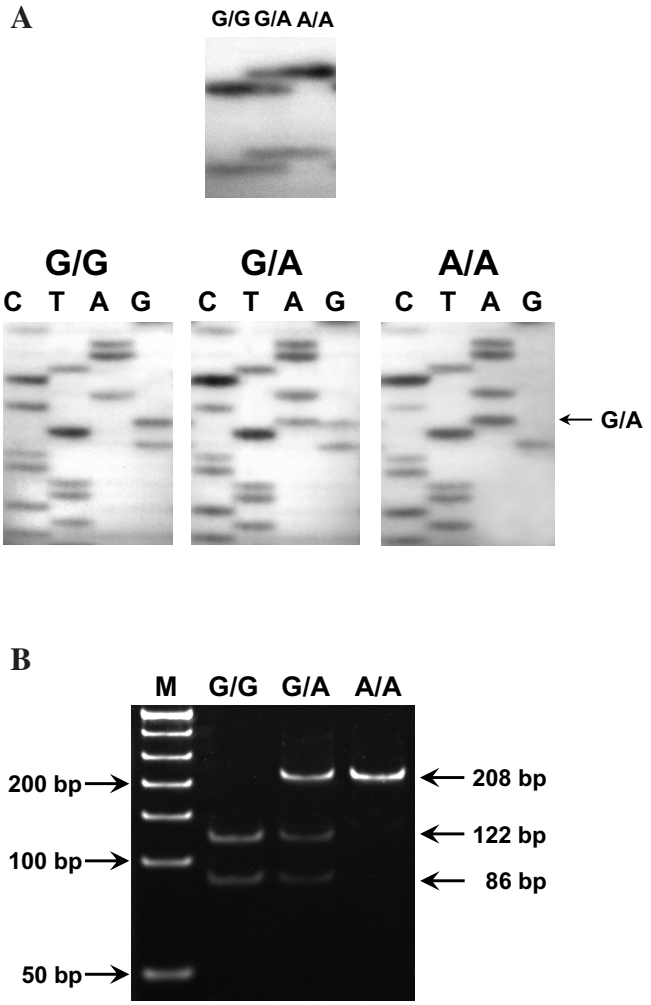

Figure 1. Genotypes analysis at IVS1-27 G/A polymorphism site of the *KLF6* gene: SSCP band patterns and sequencing of homozygote G/G and A/A, and heterozygote with G/A (arrow) (A); the PCR products were digested with *Bas* AI restriction endonuclease. If the recognition site was present, amplified DNA (208 bp in size) produced two fragments, 86 bp and 122 bp (B).

were uncommonly found in gastric cancers [6], suggesting that additional molecular mechanism of KLF6 inactivation contributes to the gastric cancer development or progression.

In the present study, we investigated whether the *KLF6* IVS1 -27G/A polymorphism of *KLF6* 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 cancers.

Materials and methods

Tissue samples. Archival normal gastric mucosa specimens from 264 gastric cancer patients who had undergone surgery at the College of Medicine, The Catholic University of Korea in Seoul, between 2000 and 2003 were enrolled in this study. All gastric cancers were pathologically confirmed as stomach adenocarcinomas. The 264 cases included 173 men (65.5%) and 91 women (34.5%) with a median age of 59 (22-85) years at initial diagnosis. Histologically, the cancers consisted of 142 intestinal-type (53.8%) and 122 diffuse-type (46.2%) gastric cancers. The healthy control population was collected from the same geographic region and consisted of 164 males and 135 females with a mean age of 44. 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 [16]. 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, Wilmingon, DE, USA).

SSCP, DNA sequencing and RFLP. Genomic DNAs from gastric mucosal tissues and blood were amplified with primers covering the *KLF6* polymorphism in intron 1. For polymerase chain reaction (PCR), the primer sequences were as follow: Sense 5'-ccgggcagcaatgttatc-3' and antisense 5'- tttggtccacagatcttcctg-3'. Each 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 56°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. 1A). We also performed PCR-restriction fragment length polymorphism (RFLP) to confirm the PCR-SSCP results. After amplification with the same primer set, the PCR products were digested with 5 U of

Table 1. Distribution of *KLF6* genotype and allele frequency in gastric cancer patients and controls.

KLF6 genotype	Cases (n=264)		Controls (n=299)		Crude OR (95% CI)	Adjusted[a] OR(95% CI)
	Number	Percent	Number	Percent		
GG	242	91.7	275	92.0	1.00	1.00 0.67 9.46
GA	15	5.7	21	7.0	0.81 (0.41-1.61)	(0.27-1.67)
AA	7	2.6	3	1.0	2.65 (0.68-10.36)	(1.71-52.25)
A allele frequency [b]	0.055		0.045			
Trend test [c]					*P*=0.5132	*P*=0.1685

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

[b] Two-sided χ^2– test: for allele frequencies, $P = 0.4515$; for genotype distribution, $P = 0.2808$

[c] Calculated in the logistical regression model using the number of A alleles in the genotypes as a continuous variable.

Table 2. Stratification analysis of *KLF6* genotype frequency in gastric cancer.

Variable	*KLF6* 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	45	2	4	225	17	2	0.58 (0.13-2.61)	9.54 (1.67-54.4)
>50	197	13	3	50	4	1	0.90 (0.28-2.92)	0.97 (0.10-9.73)
Sex								
Male	163	8	2	154	10	0	0.64 (0.18-2.24)	4.72 (0.23-99.20) [b]
Female	79	7	5	121	11	3	0.71 (0.19-2.57)	6.73 (1.19-38.22)
Lauren's								
Diffuse	112	7	3	$P = 0.9834$ (χ^2– test)				
Intestinal	130	8	4					

[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.

[b] Unadjusted OR (95% CI) adding 0.5 to all response categories due to zero cell.

restriction enzyme Bsa Al at 37 °C for 16 hr. DNA fragments then were electrophoresis on a 12% polyacrylamide gel (Fig. 1B).

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 (CI).

Results

The genotype frequencies of IVS1 -27G/A SNP of the *KLF6* in Korean gastric cancer cases and controls are summarized in table 1 and 2. The frequencies of genotype G/G, G/A and A/A in normal healthy individuals were: 91.9%, 7.0% and 1.1%, respectively. Unexpectedly, only 3 healthy controls carried the rare IVS1-27A allele at homozygosity. The frequencies of G and A alleles were 95.5% and 4.5% in healthy control individuals, respectively. However, genotype frequencies of all groups were in accordance with those previously reported in the Caucasian population [9, 10, 11]. Statistically, there were no significant differences in genotype and allele frequencies of the polymorphisms between male and female healthy controls (Chi-Square test, *P=0.12* and *P=0.057*).

For the gastric cancer patients, the G/G, G/A, and A/A genotypes had a prevalence of: 91.7% (242/264), 5.7% (15/264) and 2.6%, respectively, and the frequencies of G and A alleles were 94.5% and 5.5%. The frequency of A allele was also very similar to that of Western population [10]. Interestingly, there was a statistically significant difference in the allele frequencies in comparisons between male and female gastric cancer patients (Chi-Square test, $P = 0.005$). However, the frequency of the G-allele carrier did not differ statistically between male gastric cancer patients and healthy controls (Chi-Square test, $P = 0.76$). Finally, we did not observe a significant difference in the genotypes and allele frequencies between the gastric cancer patients and healthy controls.

Discussion

Genetic factors, including polymorphisms of genes involved in tumorigenesis, may partly explain the difference in individual susceptibility to cancer [17]. Recently, Narla et al [9] has reported that a single intronic SNP of the *KLF6* gene results in increased splice variants, which functionally antagonize wild type KLF6's growth suppressive properties by affecting expression levels of the cyclin-dependent kinase inhibitor p21. In addition, IVS1 -27G/A SNP of the *KLF6* is associated with familial and sporadic prostatic cancers [9]. A potential involvement of *KLF6* polymorphisms in lung cancer risk has been

also demonstrated [10]. However, the association between the polymorphism and increased risk of prostate cancer was not found in Ashkenazi Jewish and Finnish populations [11, 12]. In the present study, the polymorphism of the *KLF6* was not associated with increased risk of the gastric cancer in Korean. Our findings are concordant with the results of these populations. Although it is possible that IVS1 -27G/A SNP of the *KLF6* influences susceptibility to gastric cancer in population specific manner, our results suggest that the SNP polymorphism of the *KLF6* may not contribute to the gastric cancer susceptibility in Korean.

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 [18]. Although both types seem to start from *H. pylori*-related chronic gastritis [19], epidemiologic and histopathological evidence have shown that gastric carcinoma may be influenced by genetic polymorphisms, age of cancer onset and gender. Thus, we also analyzed histological subtype of gastric cancer to see whether the A-allele associate with cancer cell differentiation. Finally, there was no statistically significant association between the A-allele and histologic subtype of gastric cancer (Chi-Square test, $P = 0.878$).

In conclusion, the present study demonstrated that gastric cancer does not appear to be associated with the IVS1 -27G/A polymorphism of the *KLF6* gene in a Korean population. In addition, we found no association of gastric cancer with the polymorphism studied when patients were sorted by histological type. More work is needed to determine the significance of *KLF6* polymorphism in a large population and to define the effect of *KLF6* on gastric cancer susceptibility.

Cho Yong Gu and Hwa Sung Lee contribute equally to this work.

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