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The *COX-2*-1195AA Genotype Is Associated with Diffuse-Type Gastric Cancer in Korea

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Background/Aims: The potential role of the cyclooxygenase (COX)-2 polymorphism has been reported in relation to the risk of gastrointestinal tract malignancies. Therefore, we investigated whether COX-2 polymorphisms are associated with the risk of gastric cancer (GC) in Korea, one of the areas with a high prevalence of this condition. **Methods:** We evaluated the genotypic frequencies of *COX-2*-765 and -1195 in 100 peptic ulcer patients, 100 GC patients, and 100 healthy controls. The polymorphisms of the *COX-2*-765 and -1195 genes were analyzed by polymerase chain reaction and restriction fragment length polymorphisms. **Results:** The frequencies of the *COX-2*-1195 GG, GA, and AA genotype were 20%, 60%, and 20% in intestinal-type GC and 8%, 48%, and 44% in diffuse-type GC, respectively (p=0.021). There were no significant differences in the frequency of *COX-2*-765 genotypes between intestinal-type GC and diffuse-type GC (p=0.603). Age- and sex-adjusted logistic regression analysis showed that the *COX-2*-1195 AA genotype was the independent risk factor of diffuse-type GC compared with the *COX-2*-1195 GG genotype (p=0.041; odds ratio, 6.22; 95% confidence interval, 1.077 to 35.870). **Conclusions:** The *COX-2*-1195 AA genotype may render subjects more susceptible to diffuse-type GC. **(Gut Liver 2012;6:321-327)**

Key Words: Stomach neoplasms; Diffuse type; *COX-2*; Polymorphism

INTRODUCTION

Although *Helicobacter pylori* infection has been generally accepted as the main risk factor for gastric cancer (GC), carcinogenesis of GC is still unclear.[1,2] *H. pylori* infection alone is not enough to explain the gastric carcinogenesis because GC develops in only a small portion of infected subjects. It has been suggested that GC may have a more complex mechanism involving bacterial, dietary, and host factors that are intimately interconnected. Among the host factors, single nucleotide polymorphisms (SNPs) of interleukin (IL)-1, IL-10, and tumor necrosis factor (TNF)-α have been thought to play an important role in Caucasians.[3-5] The impact of polymorphisms of IL-1, IL-10, and TNF-α on the development of GC, however, is still controversial in Asians including our previous studies.[6-9] Recently, several studies suggested that cyclooxygenase (COX)-2 polymorphisms are related with the high risk of various human malignancies, predominantly in gastrointestinal tracts including GC.[10-14] As well known, COX is a rate-limiting enzyme that converts arachidonate to prostaglandins. As of today, three isoenzymes COX-1, COX-2, and COX-3 were found in human.[15] Among them, COX-2, only expressed by various stimuli such as cytokines, growth factors, and mitogens, is responsible for inflammatory process and carcinogenesis. In particular, increased COX-2 expression is linked to progression of gastric pre-malignant lesions and gastric carcinogenesis by activating angiogenesis, inhibiting apoptosis, and accelerating invasion and metastasis.[16-19] Moreover, it has been reported that SNPs in the promoter region of the COX-2 encoding gene have a direct effect on COX-2 expression and its functional activity. That is, *COX-2*-765 C allele increased the production of PGE_2 and PGD_2 in asthma,[20,21] and *COX-2*-1195 A allele is associated with increased COX-2 expression in esophageal cancer.[22] In GC, to our knowledge, few studies have been published on the SNPs of the promoter region -765 (rs689466) or -1195 (rs20417) of the COX-2 encoding gene.[10-13] Moreover, it is still inconclusive although it is probable that those polymorphisms are helpful to identify the high-risk subjects for GC

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development.[23]

GC can be subdivided into two pathologic entities with intestinal and diffuse types, which have distinct epidemiological and clinical features. It is generally accepted that the carcinogenesis of intestinal type GC shows a multi-step progression of gastric mucosal lesions from atrophic gastritis to adenoma, followed by dysplasia and then eventually GC.[24] To date, few data about polymorphisms of sporadic diffuse type GC has been reported.[25] GC is highly prevalent in the Far East, especially in Korea, Japan, and China. According to a recent survey, in South Korea, GC incidence in men and women reaches 62.8 and 25.7 cases per one hundred thousand, respectively.[26] Such a high incidence of GC in South Korea remains an etiologic and treatment challenge. Therefore, in this study, we focused whether COX-2 polymorphisms are associated with risk of GC, *H. pylori* infection, and pathologic types of GC in South Korea, one of the most prevalent areas of GC in the world.

MATERIALS AND METHODS

1. Patients and samples

A total of three hundred cases with peptic ulcer diseases (PUDs) (n=100), GC (n=100), and healthy controls (n=100) were enrolled and analyzed. Healthy controls were recruited voluntarily, who were conducted a routine medical check-up at Hallym University Kangdong Sacred Heart Hospital, Seoul, South Korea. All enrolled subjects underwent endoscopy, and the endoscopic findings were reviewed by two experienced endoscopists with a blind fashion. The control group has superficial gastritis or normal appearance of the gastric mucosa endoscopically. PUD and GC were diagnosed by endoscopic findings combined with histology. GC was subdivided into two distinct pathologic entities according to the Lauren's classification by an experienced pathologist blindly. The tumor, node, metastasis (TNM) stages were assigned according to the 6th American Joint Committee on Cancer TNM staging system.[27] GC was grouped into cadiac and non-cadiac cancer by the locations of GC. *H. pylori* infection was evaluated by histologic examination, rapid urease test, and/or anti-*H. pylori* immunoglobulin G quantification (GCRL Co., Seoul, Korea; sensitivity, 98.10%; specificity, 90.82%). We defined *H. pylori* infection as being positive when at least one test of them showed positivity. All the subjects had no past history of *H. pylori* eradication. Buffy coat was separated from the whole blood and stored immediately at -70°C until use. The study protocol was approved by Institutional Review Board at Hallym University Kangdong Sacred Heart Hospital.

Table 1. Primers Used for the Polymerase Chain reaction

Position	Primer set	Sequences
-765 *COX-2*	Sense	5'-AGG CAG GAA ACT TTA TAT TGG-3'
	Antisense	5'-ATG TTT TAG TGA CGA CGC TTA-3'
-1195 *COX-2*	Sense	5'-CCC TGA GCA CTA CCC ATG AT-3'
	Anisense	5'-GCC CTT CAT AGG AGA TAC TGG-3'

COX, cyclooxygenase.

2. Analysis of *COX-2* gene polymorphism

Genomic DNA was extracted from the buffy coat using a commercialized kit (QIAamp; QIAGEN, Valencia, CA, USA). Following polymerase chain reaction (PCR) amplification using the primers as listed in Table 1, the COX-2 polymorphism was analyzed by restriction fragment length polymorphism (RFLP) method. Briefly, PCR conditions for *COX-2*-765 were as follows: 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute (denaturation), 60°C for 1 minute (annealing), 72°C for 1 minute (extension), and finally 72°C for 10 minutes. And then, the PCR products were digested with *Aci*I (Promega, Madison, WI, USA) at 37°C for 4 hours and separated by electrophoresis on a 2% agarose gel. A fragment containing the *Aci*I polymorphic site at position -765 of *COX-2* gene was separated as follows: the G

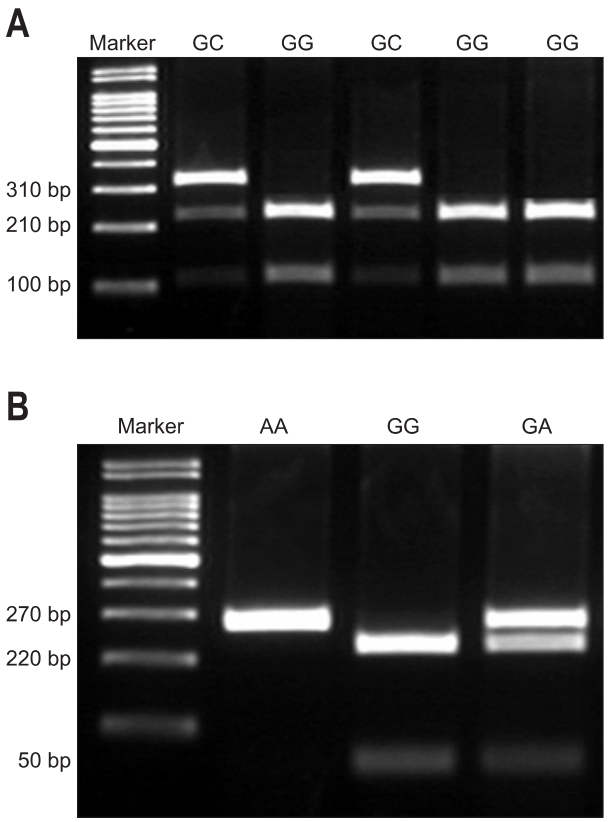

Fig. 1. Restriction patterns for cyclooxygenase (*COX*)-*2*-765 with *Aci*I and *COX-2*-1195 with *Pvu*II. (A) The genotypes of *COX-2*-765 were designated as follows: GG, 2 bands of 100/210 bp; GC, 3 bands of 100, 210, and 310 bp; and CC, a single band of 310 bp. A 100-bp ladder is displayed in Lane 1 (Marker). Lanes 2, 3, 4, 5, and 6 are GC, GG, GC, GG, and GG, respectively. (B) The genotypes of *COX-2*-1195 were designated as follows: GG, 2 bands of 50/220 bp; GA, 3 bands of 50, 220, and 270 bp; and AA, a single band of 270 bp. A 100-bp ladder is displayed in Lane 1 (Marker). Lanes 2, 3, and 4 are AA, GG, and GA, respectively.

allele was designated if two bands of 100 and 210 bp were obtained, and the C allele was designated if a single band of 310 bp was obtained (Fig. 1A).

The PCR conditions for the *COX-2*-1195 polymorphism were as follows: 94°C for 2 minutes, then 35 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, and finally 72°C for 10 minutes. A fragment containing the *Pvu*II (Promega, Madison, WI, USA) polymorphic site at position -1195 of *COX-2* gene was separated as follows: the G allele was designated if two bands of 220 and 50 bp were obtained, and the A allele was designated if a single band of 270 bp was obtained (Fig. 1B).

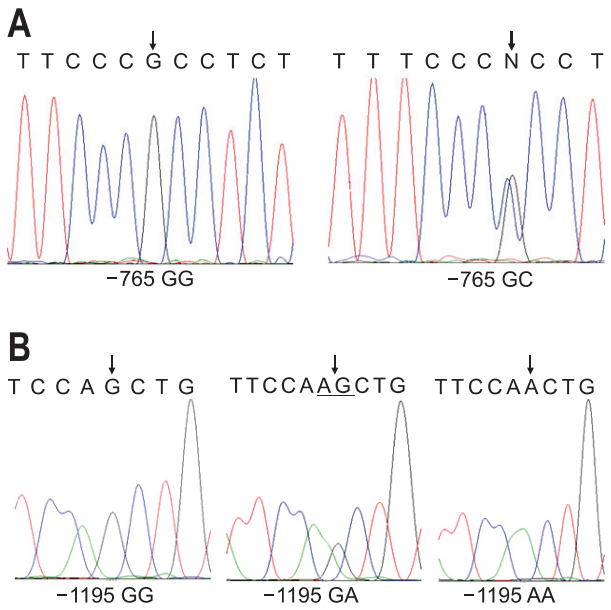

Fig. 2. Sequences of the cyclooxygenase-2 promoter region. (A) The single nucleotide polymorphism (SNP) located on nucleotide -765 is represented by arrows. (B) The SNP located on nucleotide -1195 is represented by arrows.

3. DNA sequencing of PCR products to validate RFLP

Ten samples were randomly selected for DNA sequences analysis from each group. Following PCR for *COX-2*-765 and -1195, DNA sequences were analyzed by automated DNA fluorescent sequencer using ABI prism™ Bigdye™ terminator cycle sequencing Ready reaction kit version 3.1 and ABI 3730XL capillary DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The deduced sequences were compared with the sequences from the Genbank database in order to confirm the genotype of -765 and -1195 *COX-2* genes (Fig. 2A and B).

4. Statistical analysis

The Hardy-Weinberg equilibrium of alleles at individual loci was assessed by chi-square test. Univariate analysis was performed with chi-square test and Fisher's exact test for the comparison of genotype frequencies among the groups. Age and sex adjusted odds ratios (ORs) with 95% confidence intervals (CIs) for genotyping were calculated by logistic regression analysis. The 2-sided p-value less than 0.05 was considered statistically significant. All statistical analyses were carried out using dBSTAT for Windows version 4.0 (dBSTAT Co., Seoul, Korea).

RESULTS

1. Clinical characteristics of enrolled subjects

Baseline characteristics were listed in Table 2. Mean age was significantly older in GC group than in PUD and control groups (p<0.0001). Female-to-male ratio was significantly different among the groups (p=0.03). Prevalence of *H. pylori* infection was 47%, 39.6%, and 35.9% in control, PUD, and GC group, respectively. There was no difference in the prevalence of *H. py-*

Table 2. Clinical Characteristics and Genotypic Frequencies of -765 and -1195 *COX-2* in the Subjects Enrolled

Characteristic	Control (n=100)	Peptic ulcer (n=100)	Gastric cancer (n=100)	Total (n=300)	p-value
Age, mean (range), yr	44.2 (26-79)	58.0 (23-90)	64.8 (26-87)	55.7 (23-90)	<0.0001
Sex, female/male	38/62	24/76	17/83	79/221	0.03
HP positive, %	47/100 (47)	38/96 (39.6)	28/78 (35.9)	113/278 (40.6)	0.302
-765 *COX-2*					0.265
GG	90	86	82	258	
GC	10	14	18	42	
CC	0	0	0	0	
G:C	0.95:0.05	0.93:0.07	0.91:0.09	0.93:0.07	0.293
-1195 *COX-2*					0.256
GG	22	18	14	54	
GA	41	54	54	149	
AA	37	28	32	97	
G:A	0.425:0.575	0.45:0.55	0.41:0.59	0.428:0.572	0.716

HP, *Helicobacter pylori*; COX, cyclooxygenase; GC, gastric cancer.

lori infection among the groups (p>0.05).

2. *COX-2*-765 and -1195 genotype frequencies of study groups

Genotypic frequencies of the individual loci were in the Hardy-Weinberg equilibrium. The genotypic and allelic frequencies of *COX-2*-765 and -1195 were summarized in Table 2. No *COX-2*-765 CC genotype was found. There were no significant differences in the distribution of *COX-2*-765 and -1195 genotypes among the groups (p=0.302).

3. *COX-2*-765 and -1195 genotype frequencies according to the pathologic type of GC

We summarized the baseline characteristics of the patients with GC subdivided by Lauren's classification in Table 3. GC was confirmed as having diffuse type in 50 (50%) cases and intestinal type in 50 (50%) cases. There were no differences in the distribution of age, sex, smoking, and the prevalence of *H. pylori* infection between intestinal type GC and diffuse type GC. Significant difference was observed in the distribution of TNM stages between two groups (p<0.0001). Advanced GCs (AGCs) were observed more frequently in diffuse type GC (73.9% vs 43.8%, p=0.003). The location of GC was significantly different between the two groups (p=0.007). All the cardiac GC was diffuse type. There were significant differences in the frequency of *COX-2*-1195 genotypes between two groups (p=0.021). However, no significant differences were found in the frequency of *COX-2*-765 genotypes between two groups.

4. Risk of intestinal or diffuse type GC according to genotype of *COX-2*-1195

Comparing control group with diffuse type GC group, the age and sex adjusted logistic regression analysis showed that the subjects with *COX-2*-1195 AA genotype had the OR of 6.22 for diffuse type GC compared with the subjects carrying the *COX-2*-1195 GG genotype (p=0.041; 95% CI, 1.077 to 35.870). Comparing intestinal type GC with diffuse type GC, the age, sex, and smoking adjusted OR for *COX-2*-1195 AA genotype relative to GG genotype was 5.55 (p=0.001; 95% CI, 2.044 to 15.065) (Table 4).

Table 3. The Clinicopathologic Characteristics and Genotypic Frequencies of -765 and -1195 *COX-2* according to the Histological Type of Gastric Cancer

Characteristic	Gastric cancer		p-value
	Intestinal type (n=50)	Diffuse type (n=50)	
Age, mean (range), yr	65.5 (47-87)	64.1 (26-87)	0.574
Sex, female/male	7/43	10/40	0.424
Smoker	15 (30)	8 (16)	0.096
HP positive	12/44 (27.3)	16/34 (47.1)	0.071
TNM stage	48	46	<0.0001
0	8 (16.7)	0 (0)	
Ia	19 (39.6)	12 (26.1)	
Ib	0 (0)	4 (8.7)	
II	2 (4.2)	10 (21.7)	
IIIa	6 (12.5)	4 (8.7)	
IIIb	2 (4.2)	0 (0)	
IV	11 (22.9)	16 (34.8)	
≥Ib	21 (43.8)	34 (73.9)	0.003
Location			0.007
Noncardia			
Antrum	29 (58)	28 (56)	
Body	21 (42)	14 (28)	
Cardia	0 (0)	8 (16)	
-765 *COX-2*			0.603
GG	40 (80)	42 (84)	
GC	10 (20)	8 (16)	
CC	0	0	
G:C	0.9:0.1	0.92:0.08	
-1195 *COX-2*			0.021
GG	10 (20)	4 (8)	
GA	30 (60)	24 (48)	
AA	10 (20)	22 (44)	
G:A	0.5:0.5	0.32:0.68	

Data are presented as number (%).
HP, *Helicobacter pylori*; TNM, tumor, node, metastasis; COX, cyclooxygenase; GC, gastric cancer.

Table 4. The Risk of Intestinal- or Diffuse-Type Gastric Cancer according to the Genotype of -1195 *COX-2*

-1195 *COX-2* genotype	Control vs intestinal type GC		Control vs diffuse type GC		Intestinal vs diffuse type GC*	
	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value
GG	1.00		1.00	-	1.00	-
GA	1.24 (0.277-5.558)	0.779	4.03 (0.717-22.689)	0.113	2.04 (0.821-5.047)	0.125
AA	0.40 (0.075-2.079)	0.274	6.22 (1.077-35.870)	0.041	5.55 (2.044-15.065)	0.001

Adjusted for age and sex.
COX, cyclooxygenase; GC, gastric cancer; OR, odds ratio; CI, confidence interval.
*Adjusted for age, sex, and smoking.

Table 5. Gastric Cancer Risks according to *Helicobacter pylori* Infection and *COX-2*-1195 Genotype Frequencies

HP status	-1195 *COX-2* genotype	No. (C/I/D)	Control vs intestinal type GC		Control vs diffuse type GC	
			OR (95% CI)	p-value	OR (95% CI)	p-value
Negative	GG	14/6/2	1.00		1.00	-
Negative	GA	30/21/12	1.6 (0.540-4.942)	0.428	2.8 (0.551-14.231)	0.308
Negative	AA	14/5/4	0.8 (0.206-3.376)	1.000	2.0 (0.314-12.745)	0.660
Positive	GG	4/2/2	1.2 (0.166-8.186)	1.000	3.5 (0.368-33.308)	0.541
Positive	GA	20/5/4	0.6 (0.148-2.294)	0.5	1.4 (0.225-8.724)	1.000
Positive	AA	14/5/10	0.8 (0.206-3.376)	0.798	5.0 (0.923-27.078)	0.079

COX, cyclooxygenase; HP, *Helicobacter pylori*; C, control; I, intestinal type gastric cancer; D, diffuse type gastric cancer; GC, gastric cancer; OR, odds ratio; CI, confidence interval.

5. GC risks according to *H. pylori* infection and *COX-2*-1195 genotype frequencies

There were no significant differences in the distribution of *COX-2*-1195 genotypes and status of *H. pylori* infection between control and GC (Table 5).

DISCUSSION

The role of genetic backgrounds for diffuse type GC remains to be clarified. In addition, it is still unknown whether diffuse type GC develops through a multi-step progression like intestinal type GC. In the present study, for the first time, we revealed that *COX-2*-1195 AA genotype was the independent risk factor of diffuse type GC in the prevalent area of GC.

Although the carcinogenesis of diffuse type GC in relation to COX-2 polymorphism is unknown, a few studies suggested the possible role of COX-2 polymorphism in gastric carcinogenesis. Liu *et al*.[11] reported the significant association between COX-2 overexpression and the -1195 AA genotype in pre-malignant gastric lesions. Another study demonstrated that the -1195 A allele creates a C-MYB binding site, a transcriptional factor related in the regulation of the balance among cell division, survival, and differentiation,[28] resulting in a higher transcriptional activity of the *COX-2* gene.[23] Additionally, COX-2 overexpression increases adhesion to extracellualr matrix proteins, and inhibits programmed cell death by reduction of transforming growth factor β2 receptor and E-cadherin levels, down-regulation of which is associated with diffuse-type GC.[29-33] Taken together, COX-2 overexpression may be involved in intestinal epithelial cell differentiation, apoptosis, and local invasion of tumor cells.

However, the *COX-2*-1195 gene polymorphism in our study did not increase the overall risk of GC, which differs from the Chinese study.[11] The relatively high frequency of *COX-2*-1195 AA genotype in our control group could explain the reason why the genotypic frequencies of *COX-2*-1195 were not different between control and GC group. In fact, the frequency of *COX-2*-1195 AA genotype in our control group (37%) was similar to that in GC group (35.5%) of the Chinese study. Considering that the frequency of *COX-2*-1195 AA genotype in our control group (37%) was higher than that of Chinese study (23.7%), it might be due to the different distribution between two populations. Although the number of our control group was relatively small, the frequencies of *COX-2*-1195 genotype in this study were very similar to the results from the large population based study of rheumatoid arthritis in Korea that included 1,000 subjects.[34] Therefore, the present study suggests that a certain type of COX-2 polymorphism can be considered as a risk factor for diffuse-type GC in Korea.

The frequencies of *COX-2*-765 genotype were not different among the groups in our study. *COX-2*-765 C carriers have been reported to have 3-8 fold increased risk of GC in a few studies,[10,12] while its polymorphism was not associated with GC in the other studies.[11,13] These conflicts may come from the different genotypic frequencies among ethnic populations as well as the different environmental, dietary, and *H. pylori* virulent factors. For instance, only 5% of control group in our study and Chinese population had *COX-2*-765 C allele,[11] while 22% in Portuguese[10] and 16% in Northern Indian did.[12]

In this study, sub-analysis was carried out to investigate the interaction between *H. pylori* infection and *COX-2* polymorphism. No significant difference, however, was observed in the distribution of genotypic frequencies of *COX-2* polymorphism and status of *H. pylori* infection between control and GC. Liu *et al*.[11] reported that *H. pylori* infected tissues could stimulate the host COX-2 expression much stronger in subjects with *COX-2*-1195 AA genotype than GG genotype. However, Saxena *et al*.[12] described that *COX-2*-765 C carriers were susceptible to developing GC, which were out of relation to *H. pylori* infection. Therefore, the degree of the contribution of *H. pylori* infection, even though it is classified as a definite carcinogen, on the gastric carcinogenesis might vary according to ethnicity and prevalence of GC.[35,36] Putting theses previous reports together, it is thought that interaction between *H. pylori* infection and the *COX-2* polymorphism is not conclusive especially in the prevalent area of GC such as Korea. Interestingly, the prevalence of

H. pylori was lower in GC than that of expected, though there was no statistical difference between control and GC group. This result can be explained by the results of some reports, which showed a high rate of false negative for detecting current *H. pylori* infection by rapid urease test or histology in atrophic and/or metaplastic gastric mucosa.[37-39] Recent Korean study showed *H. pylori*-positive rates of 24% in antrum, 50% in gastric body by rapid urease test in metaplastic gastric mucosa.[39]

In conclusion, it suggests that *COX-2*-1195 AA genotype may make the subjects more susceptible to diffuse type GC in Korea. However, GC risk according to *H. pylori* infection and *COX-2*-1195 genotype frequencies was not conclusive, because there were many subgroups according to *H. pylori* infection status and COX-2-1195 genotypes, resulting too small number of subjects in each group to be compared with statistical significance. Considering the epidemiological, clinical, and genetic differences between intestinal and diffuse type GC, larger study including various host factors, bacterial factors, and ethnics will be warranted in the future.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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