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FULL PAPER

Heterogeneity of *Helicobacter pylori bab* genotypes and their association with clinical outcomes in Korean gastroduodenal patients

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SUMMARY

In this study, we aimed to investigate the prevalence of *bab* genes (*babA*, *babB*, *babC*) at their three loci (loci A, B, and C) in *Helicobacter pylori* strains from varied clinical manifestations of Korean gastroduodenal patients.

The overall prevalence of *H. pylori* Korean strains positive for *babA* and *babB* was 91.1% and 92.2%, respectively, but all strains were negative for *bab C*. *H. pylori* strains with two loci occupied (loci A and B) were the most prevalent in Korean patients (85.6%), compared to one locus occupied (14.4%) (locus A or B). Twelve *bab* genotypes were detected, additionally, the distribution of three *bab* genotypes was significantly associated with different clinical outcomes among Korean patients. The genotypes *babA*/*babB*/- and *babA*/*babA*+*babB*/- were significantly associated with peptic ulcer disease (PUD) (63.3%) and gastritis (GT) (33.3%) patients, respectively. In addition, we found that the *babA*+*babB*/*babA*+*babB*/- genotype was significantly associated with gastric cancer (GC) (36.7%) as compared to GT (6.7%) or PUD (6.7%) ($p<0.05$) patients.

This study provided evidence that the *bab* genotypes in *H. pylori* Korean strains were highly variable. Interestingly, three patterns of *bab* genotypes were significantly different among patients with different clinical outcomes in the population at high-risk for GC.

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INTRODUCTION

Helicobacter pylori is a Gram-negative microaerophilic bacterium that is the major causative agent of human gastroduodenal diseases (Ahuja and Sharma 2002). This bacterium is responsible for the development of chronic gastritis (GT), peptic ulcer diseases (PUD), and gastric cancer (GC) (Bernardini *et al.,* 2017). As chronic infection with *H. pylori* has been implicated in GC development, this bacterium is classified as the group I carcinogen by the International Agency for Research on Cancer (IARC) (1994). According to GLOBALCAN 2018 data, GC remains an important cancer worldwide: it is the fifth most numerous among all cancers and the third leading cause of cancer-related mortality (Bray *et al.,* 2018). The incidence of GC is highly variable by geographical region (Rawla and Barsouk 2019). The term Asian enigma has been applied to describe those nations experiencing a high prevalence of *H. pylori* infection but a low incidence of GC, such as Thailand, India, and Bangladesh (Sahara *et al.,* 2012). However, in East Asian countries including South Korea, GC incidence has increased markedly (Bray *et al.,* 2018). The reasons for these differences and general worldwide variability of GC may involve complex interactions between multiple factors, including differences in host genetic, environmental, and dietary characteristics, as well as genetic diversity of *H. pylori* strains. High genetic variability is one well-known characteristic of *H. pylori*. This heterogeneity may modify the expression of virulence factors that also contribute to variation in disease development (Wroblewski *et al.,* 2010). Vacuolating cytotoxin A (VacA) and cytotoxin-associated gene A (CagA) are well-characterized as effective toxins of *H. pylori* that increase the risk of severe diseases via multiple mechanisms. *In vitro* study revealed that *H. pylori* harboring the *vacA s1m1* genotype is the most cytotoxic, more than the

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vacA s1m2 or *vacA s2m2* genotype (Atherton *et al.,* 1995). It also indicated that *H. pylori* CagA plays an important role in gastric carcinogenesis: populations infected with *H. pylori* CagA East Asian strains were at greater risk for GC than those infected with CagA Western strains (Hatakeyama 2004). Our previous study revealed that the *vacA s1m1* and *cagA* East Asian genotypes were significantly more prevalent in *H. pylori* strains colonizing Korean gastroduodenal patients than Thai patients (Boonyanugomol *et al.,* 2018; Boonyanugomol *et al.,* 2020). Nonetheless, our previous findings did not find any correlation between *vacA s1m1* or *cagA* East Asian genotypes with clinical outcomes (GT, PUD, or GC) among patients at high risk for GC. However, other virulence factors of *H. pylori* have been suggested to participate in gastroduodenal pathogenesis, and their correlation with clinical outcomes should be explored.

Besides *H. pylori* effective toxins, bacterial adherence capability is the first important step for colonization in the pathogenic process of *H. pylori* infection. Blood group antigen-binding adhesin (BabA) is an outer membrane protein (OMP) of *H. pylori* that plays an important role in bacterial attachment to fucosylated Lewis b (Le^b) blood group antigens on gastric epithelial cells (Yamaoka 2008). It has been suggested that BabA-mediated attachment may facilitate CagA or its products delivery into gastric epithelial cells and stimulate inflammatory responses (Ishijima *et al.,* 2011). Two closely related paralogs to BabA have been reported, BabB and BabC, but their functions are still unknown (Pride and Blaser 2002). The *bab* family of genes could be located downstream at three chromosomal loci, such as *hypD*, *s18,* and *hp0318*, represented as locus A, B, and C, respectively (Matteo *et al.,* 2011). Several studies reported that most of the *babA* and *babB* positive *H. pylori* strains preferentially locate, respectively, at locus A and B (Colbeck *et al.,* 2006; Hennig *et al.,* 2006). However, the homologous genes *babA*, *babB,* and *babC* can be exchanged among these three loci by gene conversion through homologous recombination (Hanada and Yamaoka 2014). Previous evidence supports that *babA* and *babB* in *H. pylori* J99 and 26695 strains have been detected in reversed chromosomal locations due to recombination events (Tomb *et al.,* 1997; Alm *et al.,* 1999). Accordingly, altering *bab* genes through recombination might contribute to *H. pylori* changing its adhesion molecules for environmental adaptation. However, data regarding the association between the variability of *bab* genes at their genomic loci and clinical outcomes are currently lacking, especially for those *bab* genotypes associated with GC. In this study, we characterized the genetic heterogeneity of *H. pylori bab* homologs at three different loci to determine the *bab* genotypes in *H. pylori* strains from Korean gastroduodenal patients. We also assessed the association between *bab* genotypes and three clinical outcomes (GT, PUD, and GC) in the population at high risk for GC.

MATERIALS AND METHODS

Bacterial Strains and DNA Extraction

H. pylori strains were obtained from the *H. pylori* Culture Collection, Department of Microbiology, Gyeongsang Institute of Health Sciences, Gyeongsang National University College of Medicine, Republic of Korea. There were three groups of *H. pylori* strains, including 30 strains from patients with GT, 30 strains from PUD patients, and 30 strains from GC patients, that were also examined in our previous report (Boonyanugomol *et al.,* 2020). All *H. pylori* collection cultures were grown on Brucella agar containing 10% bovine serum and antibiotic supplements at 37°C and 10% CO_2 for 48 h. Genomic DNA was extracted from *H. pylori* using a DNA extraction and purification kit (PureDireX, BIO-HELIX, Taiwan) according to the manufacturer's instruction. The purified DNA was stored at -20°C for subsequent downstream PCR assays. This study was approved by the Institutional Human Ethics Committee of Mahidol University (COA MU-CIRB 2016/053.1804).

PCR Assay of bab Genotyping

To investigate the presence of *babA*, *babB*, and *babC*, PCR assays were performed using a locus-specific forward primer (*hypD*-locus A, *s18*-locus B, and *hp0318*-locus C) and *bab*-specific reverse primer (*babA*-R, *babB*-R, and *babC*-R) provided in previously published reports (Colbeck *et al.,* 2006; Hennig *et al.,* 2006; Kim *et al.,* 2015). The sequences of primers are listed in *Table 1*. All PCR reaction volumes were 20 µL that contained 1X ready-to-use PCR master mix (OnePCR™ Plus, GeneDireX, Taiwan), 0.5 µM of

Table 1 - Primer sequences used for PCR detection of *bab* genes at three loci-

Primer	*Primer sequences*	*Ref.*
Locus A (forward)	TTTTGAGCCGGTGGATATATTAG	(Colbeck *et al.*, 2006)
Locus B (forward)	CTTTAATCCCCTACATTGTGGA	(Colbeck *et al.*, 2006)
Locus C (forward)	ACCCTAATGGGCATGTGGTA	(Hennig *et al.*, 2006)
babA (reverse)	TTTGCCGTCTATGGTTTGG	(Colbeck *et al.*, 2006)
babB (reverse)	TCGCTTGTTTTAAAAGCTCTTGA	(Colbeck *et al.*, 2006)
babC (reverse)	GACTTGCATGATGTTTGCCGC	(Kim *et al.*, 2015)

primer, and 100 ng of DNA. PCR amplification was performed in a T100 Thermal Cycler (Bio-Rad, USA) using the following protocol: 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 56.5°C for 30 sec and 72°C for 2 min, and finally, 72°C for 5 min (Colbeck *et al.,* 2006). The PCR products were electrophoresed on 1% agarose gel and visualized by UV illumination. The PCR products of *babA*, *babB*, or *babC* approximately 2.1-2.6 kb in length were expected to locate at locus A, 1.0-1.5 kb at locus B, or 1.5 kb at locus C (Ansari *et al.,* 2017). The reference strains of *H. pylori* 26695 and J99 were used as positive control.

Statistical Analysis

SPSS software version 20 (SPSS Inc, Chicago, IL, USA) was used for statistical analyses. The association between any *bab* genotype and clinical outcome was analyzed by using chi-square test. A *p*-value less than 0.05 was considered statistically significant.

RESULTS

H. pylori 26695 and J99 strains were used as the reference strains for the PCR-based detection of *bab* genes with their respective loci (*Figure 1*). *Table 2*

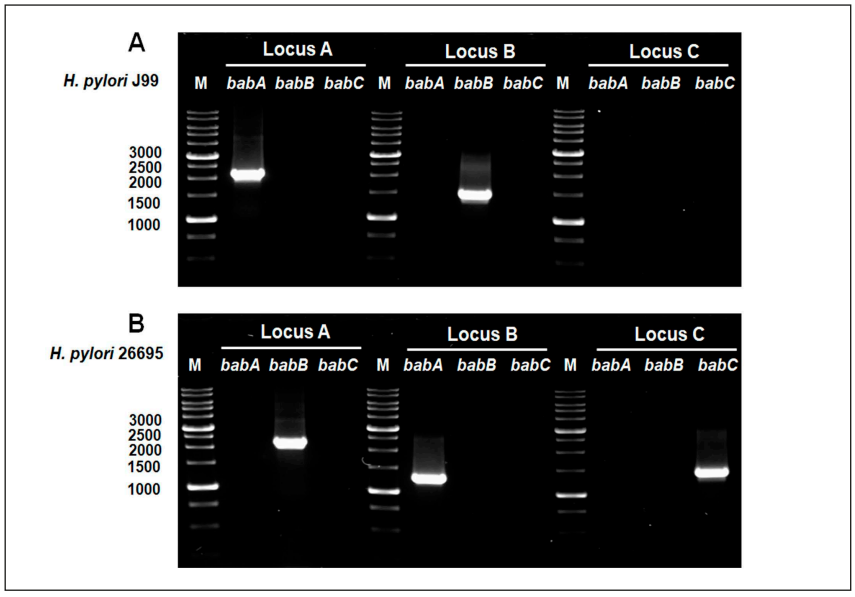

Figure 1 - *PCR amplification of bab genes at three different loci (locus A, locus B, or locus C) in H. pylori J99 (A) and H. pylori 26695 (B). PCR amplification shows H. pylori J99 strain was positive for babA and babB in loci A and B, respectively, but negative for babC with an empty locus C. Strain 26695 shows babA and babB at reversed chromosomal loci, and locus C positive for babC. A 1 kb DNA ladder is shown in lane M.*

Table 2 - *The presence of H. pylori babA, babB, and babC in Korean gastroduodenal patients.*

bab *status*	*Korean gastroduodenal patients*			
	GT (n=30)	*PUD (n=30)*	*GC (n=30)*	*Total (n=90)*
babA positive	27 (90%)	28 (93.3%)	27 (90%)	82 (91.1%)
Single *babA* positive	14 (46.7%)	23 (76.7%) [a]	13 (43.3%)	50 (55.5%)
Multiple *babA* positive	13 (43.3%) [b]	5 (16.6%)	14 (46.7%) [c]	32 (35.6%)
babA negative	3 (10%)	2 (6.7%)	3 (10%)	8 (8.9%)
babB positive	27 (90%)	28 (93.3%)	28 (93.3%)	83 (92.2%)
Single *babB* positive	23 (76.7%) [d]	23 (76.7%) [e]	11 (36.7%)	57 (63.3%)
Multiple *babB* positive	4 (13.3%)	5 (16.6%)	17 (56.6%) [f]	26 (28.9%)
babB negative	3 (10%)	2 (6.7%)	2 (6.7%)	7 (7.8%)
babC positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Single *babC* positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Multiple *babC* positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)
babC negative	30 (100%)	30 (100%)	30 (100%)	90 (100%)

Single positive refers to the presence of *babA*, *babB*, or *babC* at one locus.
Multiple positive refers to the presence of *babA*, *babB*, or *babC* at more than one locus.
[a]significant difference between PUD and GT (p=0.017) or PUD and GC (p=0.008).
[b]significant difference between GT and PUD (p=0.024).
[c]significant difference between GC and PUD (p=0.012).
[d]significant difference between GT and GC (p=0.002).
[e]significant difference between PUD and GC (p=0.002).
[f]significant different between GC and PUD (p=0.001) or GC and GT (p=0.001).

presents *H. pylori babA*, *babB*, or *babC* of Korean gastroduodenal patients within three clinical outcome categories. The overall positive status of *babA* and *babB* in *H. pylori* Korean strains was 91.1% (90% of GT, 93.3% of PUD and 90% of GC) and 92.2% (90% of GT, 93.3% of PUD and 93.3% of GC), respectively. Nevertheless, all strains were negative for *babC*.

Patterns in single or multiple *babA* or *babB* were found at chromosomal loci (*Table 2*). The strains harboring single *babA* were significantly associated with PUD (76.7%) as compared to GT (46.7%) (p=0.017) or GC (43.3%) (p=0.008). We observed a significant association between *H. pylori* strains containing multiple *babA* and GT (43.3%) and GC (46.7%) as compared with PUD (16.6%) (p<0.05). Single *babB* was significantly associated with GT (76.7%) and PUD (76.7%) as compared with GC (36.7%) (p<0.05). Additionally, *H. pylori* strains carrying multiple *babB* were also significantly associated in patients with GC (56.6%), when compared to PUD (16.6%) (p=0.001) or GT (13.3%) (p=0.001).

The distribution of the *H. pylori bab* genes at three different loci (locus A/locus B/locus C) was determined for *bab* genotyping (*Table 3*). Strains were divided into two groups, one locus occupied and two loci occupied by *bab* genes, according to the presence of *bab* genes at their three different loci (*Table 3*). However, three loci occupied or three loci empty were not found in any isolate of this study. *H. pylori* strains harboring two occupied loci (85.6%) were more frequent than those with one occupied locus (14.4%). In all strains studied, only locus A and B were occupied by *bab* genes, and locus C was empty.

Twelve *bab* genotypes were detected in strains from Korean patients (*Table 3*). We intended to consider the genotypes distributed in two loci occupied group that was more prevalent in *H. pylori* Korean strains. According to the high frequency of single *babA* and *babB* strains from PUD patients (*Table 2*), the genotype *babA*/*babB*/- was significantly associated in patients with PUD (63.3%) as compared to GT (30%) (p=0.01) or GC (16.7%) (p=0.001) (*Table 3*). We also detected a significant association between genotype *babA*/*babA*+*babB*/- and GT patients (33.3%) as compared to PUD (10%) (p=0.028) or GC (10%) (p=0.028) (*Table 3*), which is consistent with the frequency of multiple *babA*-positive strains from GT patients (*Table 2*). There was a high frequency of multiple *babA* and *babB* in strains from patients with GC (*Table 2*), and we found that the strains harboring genotype *babA*+*babB*/*babA*+*babB*/- were significantly associated in patients with GC (36.7%), when compared to GT (6.7%) (p=0.005) or PUD (6.7%) (p=0.005).

DISCUSSION

Approximately 4% of the *H. pylori* genome was found to encode outer membrane proteins (OMPs), the largest of which is the Hop family protein (Tomb *et al.,* 1997). The Hop family of proteins includes several adhesins of *H. pylori*, such as BabA, sialic acid-binding adhesion (SabA), and outer membrane inflammatory protein A (OipA), which present on the *H. pylori* cell surface and are used for bacterial attachment to the mucous layer of gastric epithelial cells (Ansari and Yamaoka 2017). BabA is well-characterized as an adhesion protein that binds *H. pylori* to the Le^b epitopes expressed on gastric epithelial cells (Yamaoka 2008). This adhesion molecule is involved not only in bacterial attachment for col-

Table 3 - *Distribution of bab genes at locus A, B, or C in H. pylori strains from Korean gastroduodenal patients.*

Locus occupied	*Distribution of bab genes*			*Korean gastroduodenal patients (n=90)*			
	Locus A	*Locus B*	*Locus C*	*GT (n=30)*	*PUD (n=30)*	*GC (n=30)*	*Total (n=90)*
One locus occupied	*babA*	–	–	3 (10%)	2 (6.7%)	1 (3.3%)	
	babB	–	–	0 (0%)	0 (0%)	2 (6.7%)	
	–	*babA*	–	0 (0%)	0 (0%)	1 (3.3%)	
	–	*babB*	–	2 (6.7%)	1 (3.3%)	0 (0%)	
	babA + babB	–	–	1 (3.3%)	0 (0%)	0 (0%)	
			Total	*6 (20%)*	*3 (10%)*	*4 (13.3%)*	*13 (14.4%)*
Two loci occupied	*babA*	*babB*	–	9 (30%)	19 (63.3%) [a]	5 (16.7%)	
	babB	*babA*	–	0 (0%)	0 (0%)	1 (3.3%)	
	babB	*babB*	–	1 (3.3%)	1 (3.3%)	1 (3.3%)	
	babA	*babA + babB*	–	10 (33.3%) [b]	3 (10%)	3 (10%)	
	babA + babB	*babA*	–	1 (3.3%)	0 (0%)	0 (0%)	
	babA + babB	*babB*	–	1 (3.3%)	2 (6.7%)	5 (16.7%)	
	babA + babB	*babA + babB*	–	2 (6.7%)	2 (6.7%)	11 (36.7%) [c]	
			Total	*24 (80%)*	*27 (90%)*	*26 (86.7%)*	*77 (85.6%)*

GT = Gastritis, PUD = Peptic ulcer diseases, GC = Gastric cancer.
[a]significant difference between PUD and GT (p=0.01) or PUD and GC (p=0.001).
[b]significant difference between GT and PUD (p=0.028) or GT and GC (p=0.028).
[c]significant difference between GC and GT (p=0.005) or GC and PUD (p=0.005).

onization, but also supports several inflammatory processes. A previous study revealed that *H. pylori babA*-positive status was linked to the development of severe complications (Ansari and Yamaoka 2017). However, the function of two paralogs closely related to BabA-BabB and BabC- is unknown (Pride and Blaser 2002). While the frequency of *H. pylori* strains containing *babA* or *babB* was high among Korean patients, no strains harboring *babC* were found. Our findings are consistent with data collected elsewhere in East Asia that demonstrated *babA2* in more than 80% of the Japanese population (Mizushima *et al.,* 2001; Con *et al.,* 2010), but revealed no association between *babA2* and clinical outcomes (Mizushima *et al.,* 2001). High frequencies of *H. pylori babA* positive strains were also detected in high- and intermediate-risk populations from Bhutan (91.8%) and Myanmar (90.7%), greater than in low-risk populations from Nepal (79.2%) and Bangladesh (70.6%) (Ansari *et al.,* 2017). Conflicting data regarding the prevalence of *H. pylori* strains containing *babA* may be due to the different geographical origin of strains (Ansari and Yamaoka 2017) or differences in the gastric ecological niches in different countries (Ansari *et al.,* 2017). Besides *babA*, evidence of detection of *babB* and *babC* genes is rarely reported, and extensive studies of *babB* or *babC* prevalence are recommended to inform comparisons between different populations.

The current study results indicate that *babA* or *babB* positive strains might be commonly distributed among populations in areas at high risk for GC, as proposed in a previous study (Ansari *et al.,* 2017). It was found that the *bab* family of genes can be located downstream at three different loci, including locus A (*hypD*), locus B (*s18*), or locus C (*hp0318*) (Matteo *et al.,* 2011). We showed that most *bab* genes of *H. pylori* Korean strains preferred to localize at either locus A or locus B but not locus C, which is in agreement with previous data from other East Asian countries (Kawai *et al.,* 2011; Sheu *et al.,* 2012). Furthermore, the proposed genetic mechanism is that these *bab* genes can be exchanged among these three loci by gene conversion through homologous recombination that might lead to diverse *bab* gene organization (Hanada and Yamaoka 2014). The occurrence of mixed *babA* and *babB* at the same locus has also been previously demonstrated (Colbeck *et al.,* 2006). Although we found twelve *bab* genotypes located at their locus A and/or locus B, only three *bab* genotypes were found association with each clinical outcome. In this study, *H. pylori* strains carrying single *babA* and *babB* (with the genotype *babA*/*babB*/-) were significantly unique in strains from PUD patients. We demonstrated that the *H. pylori* strains with multiple *babA* (at loci A and B) and single *babB* (at locus B) as *babA*/*babA*+*babB*/- genotype were significantly associated with GT patients. Notably, our study also demonstrated that the high frequency of *H. pylori* strains containing multiple *babA* and *babB* positive as *babA*+*babB*/*babA*+*babB*/- genotype was significantly associated with GC patients.

Several studies have shown *bab* genotype diversity at three different genomic loci in *H. pylori* strains. Colbeck *et al.* detected nine *bab* genotypes (at loci A and B) among 44 *H. pylori* strains (Colbeck *et al.,* 2006). In 2017, several *bab* genotypes (at loci A, B, and C) were identified in *H. pylori* clinical isolates from dyspeptic patients of Bhutan, Myanmar, Nepal, and Bangladesh; however, the strains containing two loci with the *babA*/*babB*/- genotype were the most common in each country, especially in Bhutan and Myanmar (Ansari *et al.,* 2017). Sheu *et al.* found four *bab* genotypes (at loci A and B) from 92 clinical strains of *H. pylori*. Notably, infection with the *babA*+*babB*/*babA*+*babB* genotype correlated with an increased risk of GC in a Taiwanese population, similar to the results of our current study (Sheu *et al.,* 2012). As previously mentioned, the diverse genotypes of *bab* genes located among their loci may be caused by gene conversion through homologous recombination, and this process may play an important role in the DNA repair pathways, such as in the repair of DNA double-strand breaks (DSBs) and stalled DNA replication forks (Hanada and Yamaoka 2014). The mechanism underlying homologous recombination in *H. pylori* strains may prevent genome instability from several host immune responses, including reactive oxygen species-induced DNA damage that may affect the antigenic variation of Bab proteins (Hanada and Yamaoka 2014). Authors of one study suggested that *H. pylori babA*+*babB*/*babA*+*babB* positive strains may have a better adaptation to the GC environment instead of leading to more toxicity in gastric carcinogenesis (Sheu *et al.,* 2012). Nonetheless, alteration of adhesion antigenic determinants, including Bab proteins in *H. pylori*, may be caused by host-microbe interaction. Furthermore, the plasticity in the OMP profiles of *H. pylori* strains may assist bacterial adaptation to the different niches and microenvironment changes within the human stomach during infection, such as nutrient starvation, antibiotic stresses, immunological attack, or DNA damage (Colbeck *et al.,* 2006; Hanada and Yamaoka 2014). In this study, our data showed only several patterns of *H. pylori bab* genotypes and their association with clinical outcomes in Korean patients. However, we need more exploration to assess each genotype's effects on gastric epithelial cells, including Bab protein expression controlled by phase variation, Le^b attachment ability, histological changes, levels of inflammation in gastric cells, or their signaling pathway-associated gastric carcinogenesis.

In conclusion, our study showed genetic heterogeneity of *H. pylori bab* genotypes detected in Korean gastroduodenal patients, which were recognized as a

population at high risk for GC. Interestingly, *H. pylori* infection by the mixed *babA* and *babB* at both loci A and B as *babA*+*babB*/*babA*+*babB*/- genotype was significantly associated with GC. More studies with larger sample sizes for investigating *bab* genotypes are necessary, which may lead to identifying appropriate virulence markers for evaluating disease risk. In addition, differences in other factors associated with gastric carcinogenesis should not be overlooked, including host, environmental, and dietary factors.

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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