UDC 575.
https://doi.org/10.2298/GENSR2001115O
Original scientific paper

ASSOCIATION OF INTERLEUKIN 12B RS3212227 POLYMORPHISM WITH GASTRIC CANCER, INTESTINAL METAPLASIA, AND *Helicobacter pylori* INFECTION

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Orenay-Boyacioglu S., Kasap E., Yuceyar H., and M. Korkmaz (2020). *Association of interleukin 12B RS3212227 polymorphism with gastric cancer, intestinal metaplasia, and Helicobacter pylori infection*. - Genetika, Vol 52, No.1, 115-126.

Interleukin 12 (IL-12) has a key function in promoting Th1 immune response in the gastrointestinal mucosa. Although *cytokine* gene polymorphisms are associated with increased risk of gastric cancer (GC), studies on different geographic regions and ethnic groups are not able to draw a consistent result. The current case-control study aims to find out an association between a functional *IL-12B* rs3212227 polymorphism and the susceptibility and clinical features of the study groups, which are GC, *Helicobacter pylori*–infected and *H. pylori*–uninfected intestinal metaplasia (IM). In this study, *IL-12B* rs3212227 polymorphism was genotyped in 35 GC cases, 25 *H. pylori*–infected IM patients, 25 *H. pylori*–uninfected IM patients, and 25 control subjects. PCR-RFLP analysis was performed to find out and compare the polymorphism profiles of case biopsies. There was statistical significance in genotype distributions and allelic frequencies in GC patients with proximal arrest in stomach ($p=0.042$). The rs3212227 genotypes and allelic frequencies were not correlated with any of the study groups ($p>0.05$). Other clinical features examined in the GC patients were also not correlated with the rs3212227 genotypes and allelic frequencies ($p>0.05$). Current findings suggest that *IL-12B* rs3212227 polymorphism may play a role in GC development.

Keywords: Gastric cancer, *Helicobacter pylori*, intestinal metaplasia, *IL-12B* gene, rs3212227, polymorphism.

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INTRODUCTION

The incidence and mortality rates of GC worldwide is on the decrease for more than half a century, however it is still known as one of the cancer types with the highest incidence and lethality (MOHAMMADIAN *et al*., 2018). Today, GC is the fifth leading cancer type in incidence accounting for almost a million new cases diagnosed annually (BRAY *et al*., 2018). According to Lauren's histological classification, GC is divided into two groups, the well differentiated intestinal carcinomas and the poorly differentiated diffuse-type cancers (PANANI, 2008). *H. pylori* is classified as class I carcinogen by the World Health Organization and International Agency for Research on Cancer Consensus Group (IARC GROUP, 1994). *H. pylori* is regarded as the most important risk factor for both the diffuse and intestinal types of GC (SIPPONEN *et al*., 2006). *H. pylori* infection can induce gastric inflammation and the diseases that include IM, chronic gastritis, peptic ulcer, and more rarely GC. Host susceptibility, environmental factors, and the genetic diversity of the organism might determine the progression of the infection (NAUMANN *et al*., 2004).

Proinflammatory cytokines are produced rapidly in gastric mucosa as a result of *H. pylori* infection. The cytokine IL-12 expressed by phagocytic cells, B cells, and other antigen presenting cells has a key role in regulating the inflammatory cascade network (WOLF *et al*., 1994; BIRON *et al.*, 1995). Upon an infection, inflammatory response is developed through IL-12 promotion of interferon synthesis by T cells and natural killer cells, and also the increase of Th1-type cytokine profile (MANETTI *et a*l., 1993; WU *et al.*, 1993). IL-12 is composed of p35 and p40 subunits that are encoded by *IL12A* and *IL12B* genes on chromosomal regions 5q31–33 and 3p12–3q13.2, respectively. An active IL-12 (p70) requires for both subunits to be expressed simultaneously, which are regulated at *IL-12B* transcription. The end result of the effector Th1 cell response in gastrointestinal mucosa is being determined by IL-12 (KARTTUNEN *et al*., 1997; BAUDITZ *et al*., 1999; HIDA *et al*., 1999). Sequencing studies showed that the *IL-12B* gene has intronic polymorphisms and a *TaqI* restriction fragment length polymorphism (HUANG *et al*., 2000), which result in inter-individual variations in the level of IL-12 production (SEEGERS *et al.*, 2002). Vital T-cell responses that mediate or protect against infections or immune diseases may be modified by the changes in IL-12 production. There are limited reports available that examine the correlation between the GC development and *IL-12B* rs3212227 (YIN *et al*., 2015). In the current study, we aimed to analyze the role of the *IL-12B* rs3212227 polymorphism in susceptibility and clinical outcome of GC, *H. pylori*–infected IM, and *H. pylori*–uninfected IM cases.

MATERIALS AND METHODS

Ethics statement

This study was approved by the Institutional Review Board of our university on May 20th, 2009. A signed informed consent form was obtained from each subject prior to any study-related procedures.

Cases, endoscopy, and tissue samples

The case-control study was conducted in the Departments of Gastroenterology and Medical Biology in Manisa Celal Bayar University, Faculty of Medicine from August 2012 to

May 2015. Gastric mucosa samples from upper corpus and antral regions in the lessor curvature were obtained from the subjects through endoscopic biopsy. A subset of biopsy samples was fixed in formalin, embedded in paraffin, and stained with modified toluidine blue to microscopically detect the presence of *H. pylori*. Another set of biopsy samples were immediately frozen in 0.1 M phosphate-buffered saline and stored at -80 °C for polymorphism study.

A total of 110 endoscopic biopsy samples were obtained from distal gastric carcinoma (n=35), *H. pylori*–infected IM (n=25), *H. pylori*–uninfected IM (n=25), and cancer-free control (n=25) subjects. The cancer-free control subjects were recruited among those who had applied to the gastroenterology outpatient clinic with symptoms such as epigastric pain, bloating, early satiation, fullness, epigastric burning, nausea, and vomiting but their endoscopical, radiological, and pathological examinations had been found normal. The study groups were formed and included in the current study based on the same inclusion and exclusion criteria in the studies of ORENAY-BOYACIOGLU *et al*. (2016) and ASIK-SEN *et al*. (2012). All information regarding to age, gender, personal, and familial history of the study subjects were recorded in individual interviews with the subjects.

Prevalence and duration of disease detected in routine assessments and evaluations during endoscopy of IM patients were recorded. All findings obtained during pathological evaluations of IM patient groups to investigate their possible past stomach malignancies were recorded retrospectively. Also the biopsy materials for pathological evaluations obtained during the endoscopy screening at the time of study inclusion of patients were analyzed for malignancy and the results were recorded.

Computed tomography (CT) or Positron Emission Tomography (PET)/CT was performed for grading purpose in all patients diagnosed with GC. The disease was graded according to TNM classification of American Joint Committee on Cancer (EDGE *et al*. 2010) using the CT or PET/CT results on non-operated patients (n=25) and CT, PET/CT results and post-operation pathological evaluations on operated patients (n=15). Biopsy samples and if operated, tumor differentiation of stomach resection materials of GC patients were analyzed to assess tumor type.

DNA isolation

Genomic DNA was isolated from the gastric mucosa biopsy specimens using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) according to ORENAY-BOYACIOGLU *et al*. (2016) and ASIK-SEN *et al*. (2012), and stored at -20°C. The DNA samples were quantified using Nanodrop 1000 (Nanodrop, Wilmington, DE, USA) and the DNA samples with A260/A280 ratio between 1.8-2.0 were considered satisfactory for further steps.

Genotyping

A polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) assay for the single nucleotide polymorphism (SNP) rs3212227 was performed using the primers 5′-GGGGGCTTCACTATGTTGCCCACACTGGACTAA-3′ (forward) and 5′-GAAGGCATGGATTTTTACATATGACCTTCCATG-3′ (reverse). A 10 μl PCR reaction was prepared with 1 μl of genomic DNA (20-100 ng) as template, 5 μl 5X ***FIREPol® Master Mix***

Ready to Load (12.5 mM $MgCl_2$) (Solis BioDyne, Estonia), 0.25 μl forward primer/reverse primer (0.3 μM), and 3.5 μl ultrapure water using an Eppendorf mastercycler (Hamburg, Germany). The PCR conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, then 72 °C for 7 min. The PCR products were digested using five units of *TaqI* enzyme (New England Biolabs, MA, USA) at 37 °C for 24 h and the result was visualized in a 3% agarose gel electrophoresis. The PCR product is 323 base pairs (bp), and in the presence of a *TaqI* site (the G allele), the product is cut into two fragments of 185 and 138 bp (Figure 1).

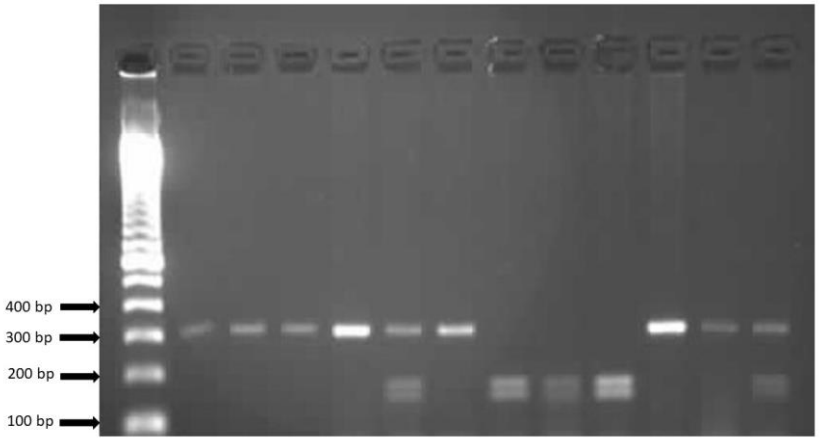

Figure 1. Genotypic representation of the *IL-12B* gene on 3% agarose gel from cases. Marker = 100 bp molecular weight marker; TT = homozygote with 323 bp; TG= heterozygote with 323 bp, 185 bp, 138 bp; GG = homozygous variable with 185 bp and 138 bp

Statistical Analysis

Sample size was determined by using the G-Power analysis program. The relationships between the study groups and their genotypes were analyzed with either Chi-squared or likelihood ratio tests. The Hardy-Weinberg equilibrium (HWE) of the groups was controlled in terms of genotypes. Descriptive statistics were given as mean and standard deviation (SD) for continuous variables and frequency and percentage for categorical variables. Statistical analyses were completed using SPSS v.11.5.1 package program with a pre-determined P value of 0.05. For analyzing the distributions of genotype and allelic data and clinical features Chi-squared test was used. We accepted two sided P values of <0.05 as significant.

RESULTS AND DISCUSSION

Although GC occurs the most frequently between the ages 50-80, it can still be seen at all ages (BOR *et al.,* 2007). The 57.2% (n=20) of the subjects in GC group of the current study

were between ages 50-80, 25.7% (n=9) was under 50 years old, and 17.1% (n=6) was over the age of 80. However, the mean age of the subjects in the control group was younger than the other three study groups. GC is usually seen more frequently in males with male/female ratio between 1.5-2.5 (LAYKE *et al.*, 2004; SHIN *et al.*, 2011). The studies conducted in Turkey also reveals a similar ratio (TUNCER *et al.*, 1992). In the current study, male/female ratio in GC subjects was calculated to be 1.7 (22/13), which could be regarded as a representative group of GC patients based on the ratios from the world and Turkish studies (BOR *et al.*, 2007). There was no significant difference between the mean age and male/female distribution of the subjects in GC, *H. pylori*–infected IM, and *H. pylori*–uninfected IM groups (p>0.05) (Table 1).

Table 1. Mean age and gender distribution of study groups

	GC (n=35)	*H. pylori*– infected IM (n=25)	*H. pylori*– uninfected IM (n=25)	Control (n=25)	P value
Mean age ±SD	54.22±13.6	51.6±12.2	52.76±14.7	46.13±10.4	0.24
Male/female subject distribution (%)	62.9/37.1	60/40	60/40	44/ 56	0.11

Table 2. Localization of tumor and distant metastasis, and survival duration features of GC patients.

A.

Tumor localization in stomach	Proximal n (%)	Distal n (%)	Diffuse n (%)
	16 (45.7)	12 (36.3)	7 (20)

B.

Distant metastasis localization	Peritoneum n (%)	Liver n (%)	Pancreas n (%)	Lung n (%)
	9 (56)	5 (32)	1 (6)	1 (6)

C.

Survival duration	<3 mo n (%)	3-12 mo n (%)
	10 (29)	8 (24)

Tumor in GC patients has been located mostly in proximal stomach (MOHAMMADIAN *et al*., 2018). In parallel with the literature, tumor localization ratios in the current study were as follows; proximal stomach 45.7% (n=16), distal stomach 34.3% (n=12), and diffuse stomach arrest 20% (n=7) (Table 2A). More than 40% of the newly diagnosed GC patients were reported to have distant metastasis (MOHAMMADIAN *et al*., 2018). The current study observed distant metastasis in 48.5% (n=16) of the subjects at the time of diagnosis based on the whole CT conducted on 33 out of 35 GC patients. Distant metastases were located to be in peritoneum in 56% (n=9), liver in 32% (n=5), pancreas in 6% (n=1), and lungs in 6% (n=1) of the patients (Table 2B). Although survival in GC is variable, 5-year survival rate is about 32%

(MOHAMMADIAN *et al*., 2018). Since our study period was 1 year, the survival rate for less than 3 mo was 29% (n=10) and for 3-12 mo was 24% (n=8) in the GC subjects of the study (Table 2C).

When the genes that regulate inflammation have polymorphisms, they generally become involved in various diseases, in which inflammation is a critical parameter (DI GIOVINE *et al*.,1992; EL-OMAR *et al*., 2000) Although the studies have detected a correlation between cytokine polymorphism and the GC risk, results are inconsistent among studies from different geographic regions and ethnical groups (ZHAO *et al*., 2012). Certain polymorphisms in cytokine genes are associated with GC. Despite the variability present across the studies, an increased GC risk is associated with *IL-1B-511*, *IL1RN* variable number of tandem repeats (VNTR), and TNFA-308 (YIN *et al*., 2015). Risk estimates have been identified based on the histological type of the tumors where associations are stronger for the intestinal type. These tumors are the most frequent ones and are followed by a set of precancerous lesions, from which IM occurs much more frequently than dysplasia and more strongly associated with GC than gastric atrophy (PELETERIO *et al*., 2010). Addressing the potential associations between the cytokine gene polymorphisms and gastric precancerous lesions may help understand some of the previous conflicting findings of studies dealing with GC as the outcome (NAVAGLIA *et al*., 2005). This is the reason for the addition of the groups *H. pylori*–infected IM and *H. pylori*–uninfected IM in the current study that scrutinizes the relation between the *IL-12* gene polymorphism and the GC risk.

IL-12 that is composed of p35 and p40 subunits encoded by *IL-12A* and *IL-12B*, respectively, directs Th1 differentiation. Th1 lymphocytes predominate over Th2 in the chronic gastritis cases associated with *H. pylori* as a first sign of *H. pylori*-associated GC. Several studies have reported elevated expression of *IL-12* mRNA and high levels of IL-12 secretion in gastric mucosa of individuals infected with*H. pylori*. In a study conducted with 110 patients with noncardia GC and 251 patients with benign gastroduodenal diseases, NAVAGLIA *et al.* (2005) reported no correlation between *IL-12* gene polymorphisms and IM; however, *IL12A* and *IL12B+15485*gene polymorphisms might affect the latest steps of gastric carcinogenesis in *H. pylori* infected subjects. GARCIA-GONZÁLEZ *et al.* (2005) reported no significant differences between peptic ulcer patients and controls in carriage, genotype, sex, and allele frequencies of *the IL-12*1188 gene polymorphism (GARCIA-GONZALEZ *et al.*, 2005) In another study conducted in Spanish population, GARCIA-GONZALEZ *et al.* (2005) could not find a relationship between cytokine polymorphisms (*IL-1B, IL-1RN, IL-12p40, LTA, IL-10, IL-4*, and *TGF-B1*) and susceptibility for GC development (GARCIA-GONZALEZ *et al.*, 2007). YIN *et al.* (2015) conducted a hospital based case-control study to evaluate the genetic effects of *IL9, IL10, IL12A, IL12B*, and *IL13* polymorphisms on the development of gastric cardiac adenocarcinoma (GCA). The rs3212227 polymorphism was highly represented in female patients and those who did not smoke or consume alcohol. The minor allele frequency of the rs3212227 polymorphism was shown 46.16% in GCA patients. These findings indicated that functional polymorphism *IL12B* rs3212227 might correlate with GCA risk. To date, this is the only study that reported an association between *IL-12B* rs3212227 polymorphism and the GCA. This association was detected in Chinese population and needs to be verified by future studies that will be conducted on other populations (YIN *et al*., 2015). In the current study, we analyzed the role of the *IL-12B* rs3212227 polymorphism in the susceptibility to and final outcome of GC disease. The

polymorphism follows the Hardy-Weinberg equilibrium. No statistically significant difference was detected between the genotype and allelic distributions of the study groups (p>0.05) (Table 3 and 4).

Table 3. Genotype distributions of the IL-12B rs32112227 polymorphism in the study groups

IL-12B rs3212227 Genotypes	*IL-12B*rs3212227 Genotype distributions n(%)				*Control-GC OR (95% CI)*	*P value*	Control-*H. pylori* infected IM *OR (95% CI)*	*P value*	Control *H. pylori* uninfected IM *OR (95% CI)*	*P value*
	GC *(n=35)*	*H. pylori* infected IM *(n=25)*	*H. pylori* uninfected IM *(n=25)*	Control (n=25)						
TT	12 (34.3)	16 (64)	11 (44)	14 (56)	1	0.16	1		1	*0.35*
TG	14 (40)	6 (24)	10 (40)	7 (28)	2.33 (0.71-7.68)		0.75(0.20-2.77)	0.67	1.82(0.52-6.33)	
GG	9 (25.7)	3 (12)	4 (16)	4 (16)	2.63 (0.64-10.73)		0.66(0.12-3.45)	0.61	1.27(0.26-6.27)	
HWE p value	0.25	0.08	0.51	0.09						

OR (95% CI): odds ratio (95% confidence interval)

Table 4. Allele frequencies of the IL-12B rs32112227 polymorphism in the study groups

IL-12B rs3212227 Allele	*IL-12B* rs3212227 Allele Frequency				*Control-GC OR (95% CI)*	*P value*	*Control-H. pylori*– infected IM *OR (95% CI)*	*P value*	*Control-H. pylori*– uninfected IM *OR (95% CI)*	*P value*
	GC *(n=35)*	*H. pylori*– infected IM *(n=25)*	*H. pylori*– uninfected IM *(n=25)*	Control (n=25)						
**T*	*0.54*	*0.76*	*0.64*	*0.70*	*1*	*0.08*	*1*	*0.50*	*1*	*0.52*
***G*	*0.46*	*0.24*	*0.36*	*0.30*	*1.96(0.91-4,22)*		*0.74(0.30-1.79)*		*1.31(0.57-3.03)*	

*Major Allele, ** Minor Allele

There was also no statistically significant difference between the *IL-12B* rs3212227 genotypes and allelic distributions in the study groups when controlled for males or females (p>0.05) (Table 5). The minor allele frequency of the *IL-12B* rs3212227 polymorphism in GC patients was 45.7% similar to that in the report by YIN *et al*. (2015). Unlike YIN *et al*. (2015) the current study did not reveal a difference in the gender distribution of *IL-12B* rs3212227 polymorphism. The sample size in our study is relatively smaller than that of YIN *et al*. (2015)

and the study populations were also ethnically different. Our findings showed a relationship between the GC patients with proximal diffuse in stomach and *IL12-B* rs3212227 polymorphism ($p<0.05$). There was no statistical significance in allele and genotype distributions in GC patients with/without diffuse arrest ($p>0.05$).Also no statistically significant differences were observed in allele and genotype distributions in both GC patient groups with distant metastasis and without metastasis ($p>0.05$). Eighteen GC patients passed away during the study and no statistically significant differences in allele and genotype distributions were detected between the dead and the surviving GC patients ($p>0.05$) (Table 6). The difference between the current results and those by YIN *et al*. (2015) may arise from the evaluation of different clinical features (present of *H. pylori* and IM, tumor localizations, distant metastasis localization, survival duration).

Table 5. Comparison of genotype distributions and allele frequencies for the IL12B rs3212227 polymorphism with gender.

IL-12B rs3212227 Genotype	Male Genotype Distributions (n)	Female Genotype Distributions (n)	OR (95% CI)	P value	*IL-12B* rs3212227 Allele Frequency	Male Allele Frequency	Female Allele Frequency	OR (95% CI)	P value
TT	20	13	1	0.98	***T**	0.55	0.50	1	0.75
TG	20	16	1.01 (0.45-2.26)		****G**	0.45	0.50	1.11	
GG	15	13	2.61 (0.54-12.79)						

OR (95% CI): odds ratio (95% confidence interval)

Table 6. Comparison of genotype distributions and allele frequencies for the IL12B rs3212227 polymorphism with arrest, metastasis and surviving|dead in GC group.

IL-12B rs3212227 Genotype	Proximal arrest (n)	Diffuse arrest (n)	Distal arrest (n)	Distant metastasis (n)	Absence of metastasis (n)	Dead GC (n)	Surviving GC (n)
TT	2	2	4	5	6	5	6
TG	7	3	4	6	6	6	6
GG	7	2	4	5	7	6	6
IL-12B rs3212227 Allele Frequency	Proximal arrest	Diffuse arrest	Distal arrest	Distant metastasis	Absence of metastasis	Dead GC	Surviving GC
*T	0.34	0.50	0.50	0.50	0.47	0.47	0.50
**G	0.66	0.50	0.50	0.50	0.53	0.53	0.50
P value	0.042***	0.17	0.31	0.26	0.37	0.35	0.23

*MajorAllele, ** MinorAllele ***Significant P<0.05

The link between the risks of GC, IM, and atrophic gastritis and the *cytokine* polymorphisms was evaluated in several studies to assess the regulatory potential of *H. pylori* infection. Polymorphisms were frequently identified in *IL-1*, *IL-10*, and *TNF-α* genes (GATTI *et al.*, 2007; SUGIMOTO *et al.*, 2007; TOGAWA *et al.*, 2005). The hypothesis stating that both host genotype and the presence of *H. pylori* may have pivotal role in the development of gastric diseases was supported in these reports. In the current study, the effect of *IL-12B* rs3212227 polymorphism on GC development was investigated for the first time in the presence of *H. pylori*, but statistically significant findings could not be obtained. This may be due to the small number of patients with the presence of *H. pylori* or the disappearance of *H. pylori* infection by treatment in some patients.

CONCLUSIONS

Despite the presence of limited number of studies showing a link between *IL-12B* polymorphisms and GC susceptibility, further studies are needed to evaluate whether other polymorphisms of *IL-12* gene or its receptors may denote susceptibility to GC. To our knowledge, this is the first study analyzing the possible link between the *IL-12B* rs3212227 polymorphism and GC, *H. pylori*–infected IM, *H. pylori*–uninfected IM. Considering the fact that cytokines form a complex interacting network, other proinflammatory and anti-inflammatory cytokine genes involved in the inflammatory response to *H. pylori* infection merit further study. The studies of cytokine gene polymorphisms might help define patient subgroups and may help lead to the development of a novel approach for the treatment of GC.

Received, March 13th, 2019
Accepted September 18th, 2019

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POVEZANOST POLIMORFIZMA INTERLEUKINA 12B RS3212227 SA GASTRIČNIM RAKOM, INTESTINALNOM METAPLAZIJOM I INFEKCIJOM *Helicobacter pylori*

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Izvod

Interleukin 12 (IL-12) ima ključnu funkciju u promovisanju Th1 imunog odgovora kod gastrointestinalne mukoze. Iako su polimorfizmi gena *cytokine* povezani sa povećanim rizikom od karcinoma želuca (GC), studije različitih geografskih regiona i etničkih grupa nisu u stanju da daju dosledan rezultat. Ova istraživanje ima za cilj da nađe vezu između funkcionalnog *IL-12B* rs3212227 polimorfizma i osetljivosti i kliničkih karakteristika ispitivanih grupa, a to su GC, *Helicobacter pylori*-inficirana i *H. pylori* – neinficirana crevna metaplazija (IM). U ovoj studiji, *IL-12B* rs3212227 polimorfizam je genotipizovan u 35 slučajeva GC-a, 25 bolesnika IM inficiranih *H. pylori*-om, 25 bolesnika sa IM *H. pylori*-nezaraznim IM i 25 kontrolnih ispitanika. PCR-RFLP analiza je izvedena da bi se utvrdio i uporedio profil polimorfizma biopsija slučaja. Bilo je statističke značajnosti u distribuciji genotipa i alelnim frekvencijama kod pacijenata sa GC proksimalnim zastojem u stomaku ($p = 0,042$). Genotipi i alelne frekvencije rs3212227 nisu bili u korelaciji ni sa jednom od ispitivanih grupa ($p > 0,05$). Ostale kliničke karakteristike ispitivane kod bolesnika sa GC takođe nisu bile u korelaciji sa rs3212227genotipovima i alelnim frekvencijama ($p > 0,05$). Ovi nalazi ukazuju da polimorfizam *IL-12B* rs3212227 može imati ulogu u razvoju GC-a.

Primljeno 13.III.2019.
Odobreno 18. IX. 2019