REVISTA DE
GASTROENTEROLOGÍA
DE MÉXICO

www.elsevier.es/rgmx

ORIGINAL ARTICLE

Helicobacter pylori genotypes associated with gastric cancer and dysplasia in Colombian patients[☆]

Y.H. Carlosama-Rosero [a,*], **C.P. Acosta-Astaiza** [b], **C.H. Sierra-Torres** [b], **H.J. Bolaños-Bravo** [b]

[a] *Grupo Interdisciplinario de Investigación en Salud-Enfermedad, Universidad Cooperativa de Colombia, Pasto, Colombia*
[b] *Grupo de Investigación en Genética Humana y Aplicada, Universidad del Cauca, Popayán, Colombia*

Abstract
Introduction and aims: Colombia has high incidence levels of gastric cancer that can be explained by the genetic variability of *Helicobacter pylori* (*H. pylori*). Our aim was to establish the relation of the *H. pylori* CagA and VacA genotypes to dysplasia and gastric cancer, in a high-risk population.
Material and methods: A case-control study was conducted on 202 patients from a high-risk cancer zone. Patients with dysplasia and gastric cancer (cases) and patients with nonatrophic gastritis (controls) were included. Endoscopic sampling and histologic classification were carried out according to the Sydney system and the Lauren classification. Genetic information was obtained through polymerase chain reaction on paraffin blocks. The measures of association of the variables of interest were evaluated in bivariate and multivariate models. A *P*<0.05 was considered statistically significant and the SPSS version 25 program was employed.
Results: Age above 50 years (OR: 23.76; CI: 8.40-67.17; *P*=0.000) and the VacA s1m1 genotype (OR: 6.18; CI: 1.25-30.51; *P*=0.025) were associated with higher risk for developing dysplasia and gastric cancer. The CagA+ genotype was not found to be a risk factor for developing those pathologies (OR: 1.02; CI: 0.39-2.62; *P*=0.965).
Conclusions: The *H. pylori* VacA genotypes are markers for the development of gastric cancer. That information could be used to create a risk index in a predictive model to optimize the healthcare of higher-risk patients.
© 2021 Asociación Mexicana de Gastroenterología. Published by Masson Doyma México S.A. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Genotipos de *Helicobacter pylori* asociados con cáncer gástrico y displasia en pacientes de Colombia

Resumen

Introducción y objetivos: Colombia exhibe elevadas tasas de incidencia de cáncer gástrico que podrían explicarse por la variabilidad genética del *H. pylori*. Nuestro objetivo fue establecer la relación de los genotipos CagA y VacA de *H. pylori* con el cáncer y la displasia en una población de alto riesgo.

Material y métodos: Estudio de casos y controles en 202 pacientes procedentes de una zona de alto riesgo de cáncer. Se incluyeron pacientes con cáncer gástrico y displasia, y pacientes con gastritis no atrófica (controles). El muestreo endoscópico y la clasificación histológica se realizaron de acuerdo al sistema de Sídney y la clasificación de Lauren. La información genética se obtuvo mediante reacción en cadena de la polimerasa sobre bloques de parafina. Se evaluaron las medidas de asociación de las variables de interés en modelos bivariados y multivariados. Un valor de $p < 0,05$ se consideró significativo. Se empleó el programa SPSS® versión 25.

Resultados: La edad mayor a 50 años (OR: 23,76; IC: 8,40-67,17; $p = 0,000$) y el genotipo VacA s1m1 (OR: 6,18; IC: 1,25-30,51; $p = 0,025$) se asociaron con mayor riesgo de desarrollar cáncer gástrico y displasia. El genotipo CagA⁺ no se mostró como un factor de riesgo para desarrollar estas enfermedades (OR: 1,02; IC: 0,39-2,62; $p = 0,965$).

Conclusiones: Los genotipos VacA de *H. pylori* son marcadores para el desarrollo de cáncer gástrico. Esta información se podría utilizar para crear un índice de riesgo en un modelo predictivo para optimizar la atención de los pacientes con mayor riesgo.

Introduction

Gastric tumors are the main cause of death by cancer worldwide. According to GLOBOCAN data for 2018, there were 1,033,701 new cases of stomach cancer, with 782,685 deaths attributed to the disease[1]. Two-thirds of stomach cancers occur in developing countries and Colombia has one of the highest incidence rates of the disease[2]. However, within the country, there are notable differences in its incidence and mortality. The coastal areas have low incidence rates, whereas they are markedly high in the Andes region and the Southwest of the country. The reasons for those differences are poorly understood, which is why some authors have called it the ''Colombian enigma''[3].

According to the Lauren classification, gastric cancer can be classified as diffuse and intestinal, with important differences in relation to epidemiology and association with premalignant lesions and *Helicobacter pylori* (*H. pylori*)[4,5].

In the intestinal histotype, the most important risk factor is *H. pylori* infection. That bacterium can produce a maladaptive response in the gastric mucosa that induces atrophy, metaplasia, dysplasia, and finally, cancer[6]. Nevertheless, only some patients with *H. pylori* infection develop cancer, leading to the postulation that genetic variability of the bacterium modulates the risk of gastric carcinogenesis. For example, the CagA+ and VacA/s1m1 genotypes have been associated with greater risk, when compared with the CagA− and VacA/s2m2 genotypes[7,8].

The majority of studies have suggested an association of the CagA and VacA s1m1 genotypes with the development of cancer, but that has been more difficult to prove in Colom-bia, given the small number of studies on the association between cancer and bacterial genotypes[9–11]. The focus of those studies has been mainly descriptive, in which allelic and genotypic frequencies are specified, reaching contradictory conclusions. Taking into account the differences in incidence of cancer in different regions of Colombia, we posit the hypothesis that the genetic variability of *H. pylori* favors the appearance of dysplasia and cancer. Our aim was to evaluate the association of the CagA and VacA genotypes of *H. pylori* with dysplasia and gastric cancer, through an analytic focus that studies the interaction of the variables in bivariate and multivariate models.

Material and methods

An analytic unmatched case-control study was conducted on patients with a histopathologic diagnosis determined from gastric biopsies at the ''*Compañía de Patólogos del Cauca*'' laboratory and the Department of Pathology of the *Universidad del Cauca*, located in Southwest Colombia. According to information from the National Administrative Department of Statistics of Colombia, the standardized mortality rates for gastric cancer in the department of Cauca are the highest in the country, at 14.7/100,000 inhabitants per year.

Through convenience sampling, our cohort included patients with a microscopic diagnosis of dysplasia and gastric adenocarcinoma (cases), patients with a histologic diagnosis of nonatrophic gastritis (controls), age 18 years and older, and patients seen at diagnostic centers during 2011. Inclusion criteria were patients with a histopathologic deter-

Table 1 Sequence of primers for PCR amplification.

Genes and regions	Sequence (5'→3')	Size (bp)
CagA	GATAACAGGCAAGCTTTTGAGG	349
VacA s1/s2	ATGGAAATACAACAAACACAC	259/286
	CTGCTTGAATGCGCCAAAC	
VacA m1/m2	CAATCTGTCCAATCAAGCGAG	570/645
	GCGTCTAAATAATTCCAAGG	

PCR: polymerase chain reaction.

mination of *H. pylori* infection, corroborated by molecular biology tests. Exclusion criteria were patients that previously presented with gastric cancer, a history of cancer, treatment for *H. pylori* infection, immunodeficiencies, and the presence of bacterial coinfection determined through polymerase chain reaction (PCR) tests.

Gastric biopsies were obtained through gastroscopy by highly experienced gastroenterologists. After an 8-h fast by the patients, topical anesthesia was applied and 5 biopsies were taken (one from the greater curvature of the antrum, one from the lesser curvature of the antrum, one from the greater curvature of the corpus, one from the lesser curvature of the corpus, and one from the incisura angularis). The biopsy specimens were fixed in 10% formalin, stained with hematoxylin-eosin, and classified using the Sydney system[12]. To control information bias, the histopathologic diagnoses were corroborated by a second pathologist and the histopathologic diagnosis of *H. pylori* infection was confirmed through PCR testing on paraffin blocks using the Chelex® technique (No. C7901; Sigma, St. Louis, MO, USA). The CagA and VacA genes were amplified through PCR, according to the method described by Sugimoto et al.[13]. The PCR mixtures were prepared with 50 ng of genomic DNA, 100 μmol dNTPs, 2.5 μl of 10X PCR buffer, 1.0 mM MgCl2, 1 U of Taq DNA polymerase (No. M1665; Promega, Madison, WI, USA), and 30 pmol of each of the primers, as shown in Table 1. Denaturalization began at 95 °C for one minute, followed by 35 denaturalization cycles at 94 °C for one minute, hybridization at 52 °C for one minute, extension at 72 °C for one minute, and a final extension step at 72 °C for 10 min. The products were analyzed by electrophoresis in 2% agarose gel at 80 volts for 40 min. The Colombian *Instituto Nacional de Cancerología* provided the *H. pylori* strains that were used as the controls. Table 1 shows the primers employed.

Statistical analysis

Because the age variable had normal distribution and the variance homogeneity hypothesis was proven, the differences between means of the groups were evaluated using the Student's t test. Differences in proportions were evaluated using the chi-square test. The odds ratio (OR) and its p value were utilized to evaluate the effect of each variable of interest on the response variable (dysplasia/gastric cancer). To assess possible interaction or confounding factors, a multivariate logistic regression test was performed. Statistical significance was set at a $p < 0.05$ and a 95% confidence interval (CI). The SPSS version 25 program was employed.
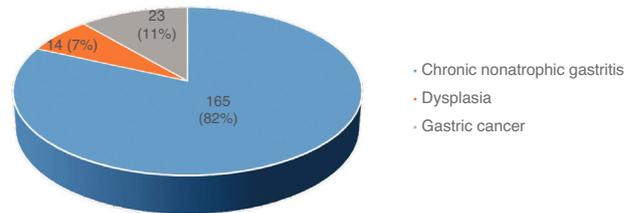

Figure 1 Patient distribution according to histopathologic diagnosis (n and percentages).

Ethical considerations

The study participants signed statements of informed consent. Ethical approval was provided by the Research Committee of the *Universidad del Cauca*.

Results

From a total of 654 patients diagnosed at referral centers, 202 were included in the study. Fig. 1 shows patient distribution by diagnosis.

Due to the fact that dysplasia and cancer are more frequent after 50 years of age, the patients were categorized into two group (patients 18 to 50 years of age and patients above 50 years of age).

Table 2 shows the distribution of patients by age, sex, and bacterial genotypes.

The chi-square test was used to evaluate the differences between the diagnostic groups, and it showed significant differences between sex and age categories ($p = 0.000$), but no significant differences between frequencies of the CagA genotype ($p = 0.175$) or the VacA genotypes ($p = 0.364$).

Because the study was unmatched, and to evaluate the distribution of the genotypes between the age groups, the proportions of genotypes in the age categories were compared, finding that the distribution of the genotypes did not vary significantly by age (chi-square test for the VacA genotypes [$p = 0.835$]; chi-square test for the CagA genotype [$p = 0.610$]) (Table 3).

Finally, the patients with dysplasia and gastric cancer were grouped together and compared with the reference category (nonatrophic gastritis), obtaining a 4.4:1 control to dysplasia/cancer ratio. To evaluate the age variable, a normality test was performed for the control group (n = 165) through the Kolmogorov-Smirnov test ($p = 0.200$). For the dysplasia/cancer group (n = 37), normality was proven through the Shapiro-Wilk test ($p = 0.700$). Once the normal distribution of the data was established, the homo-

Table 2 Distribution of the variables of interest in the study groups.

		Nonatrophic gastritis n (%)	Dysplasia n (%)	Gastric cancer n (%)
Age	18-50 years	127(77)	4(28.6)	1(4.3)
	> 50 years	38(23)	10(71.4)	22(95.7)
Sex	Female	111(67.3)	7(50)	9(39.1)
	Male	54(32.7)	7(50)	14(60.9)
CagA genotype	Negative	103(62.4)	7(50)	15(65.2)
	Positive	61(37)	7(50)	8(34.8)
	s2m2	30(18.2)	1(7.1)	1(4.3)
VacA genotypes	s1m2	2(1.2)	0(0)	0(0)
	s1m1	133(80.6)	13(92.9)	22(95.7)

Table 3 Genotype distribution in the age groups.

		18 to 50 years n (%)	> 50 years n (%)
CagA genotype	Negative	80(60.6)	47(67.1)
	Positive	52(39.4)	23(32.9)
	s2m2	20(15.2)	12(17.1)
VacA genotypes	s1m2	1(0.8)	1(1.4)
	s1m1	111(84)	57(81.5)

geneity of variance was proven with the Levene's test, based on the mean, obtaining a value of p = 0.745. With that information, the decision was made to continue the analyses using parametric tests.

The Student's t test showed statistically significant differences between the mean age of the control group (41 years) and the mean age of the patients with gastric cancer and dysplasia (62 years) (p = 0.000). For the association analyses, the reference categories were age below 50 years, female sex, the CagA− genotype and the VacA s2m2 genotype. Table 4 shows that categorized age and male sex were associated with an increase in the risk for presenting with gastric cancer and dysplasia in the bivariate and multivariate models. Even though the CagA and VacA genotypes showed no significant associations with cancer in the bivariate model, they were introduced in a first logistic regression model, given their association with cancer, described in the literature. The CagA variable showed no significant changes in any of the models, and so was excluded from the final regression (crude OR for CagA+: 4.68, CI: 0.28-77.74, p = 0.282; adjusted OR: 1.02, CI: 0.39-2.62, p = 0.965. Table 4 corresponds to the final and more parsimonious regression model.

Discussion and conclusion

In the present study, a greater risk for presenting with gastric cancer, dysplasia in patients over 50 years of age was observed. Other authors have reported similar conclusions[14,15]. Those findings are consistent with the theory of carcinogenesis in multiple steps because they suggest that the atrophy-metaplasia-dysplasia-cancer sequence is related to cellular aging. Taking into account that patients with dysplasia –a recognized premalignant lesion– were included in the present study provides relevant information

for knowing the factors associated with gastric carcinogenesis, suggests that the genetic variants of *H. pylori* could utilized be as risk markers in patients with premalignant lesions, such as, dysplasia.

Likewise, those findings underline the importance of prioritizing endoscopic and histologic studies in patients above 50 years of age, in whom cancer and dysplasia are increasingly more prevalent.

In relation to sex, we found significant differences when the study groups were compared by crude rate calculations. The association of male sex with gastric cancer has been reported by several authors[16,17], concurring with the results of our study. Possible explanations are greater exposure to carcinogens, such as alcohol and tobacco, in men[18,19], and the apparent protective effect of estrogens in women[20]. However, the modification in the effect in the multivariate model was striking, with p values and confidence intervals very close to the null hypothesis. Those findings suggest that age would play a more determining role than sex in the risk for developing dysplasia or cancer, in the study population.

Although the association of the VacA genotypes with cancer has been questioned by some authors[21], in general, the majority of studies have suggested that the VacA/s1m1 genotype and the CagA genotype condition a greater risk for the development of premalignant lesions and cancer[22–24]. In the present study, we showed that the s1m1 genotype was prevalent in both the patients with gastric cancer and the controls. Nevertheless, the strains with the s1m1 genotype were higher in the patients with dysplasia and cancer, whereas the strains with the s2m2 genotype were higher in the controls. The association of the s1m1 genotype with gastric cancer in the logistic regression model was interesting. Thus, the multivariate analysis suggested that the VacA s1m1 genotype increased the risk for developing dysplasia and cancer in patients above 50 years of age (OR:

Table 4 Measures of association for age, sex, and alleles.

	crude OR	CI	p value	Adjusted OR	CI	p value
18 to 50 years	1	1	1	1	1	1
Age > 50 years	21.39	7.8-58.7	0.000	23.76[a]	8.4-67.17	0.000
Female sex	1	1	1	1	1	
Male sex	2.69	1.30-5.58	0.007	2.33[b]	0.96-5.63	0.06
VacA s2m2 genotype	1	1	1	1	1	1
VacA s1m2 genotype	NA	NA	NA	NA	NA	NA
VacA s1m1 genotype	3.94	0.89-17.32	0.069	6.18[c]	1.25-30.51	0.025

CI: confidence interval; NA: not applicable; OR: odds ratio.
[a] Multivariate regression model adjusted by sex and VacA genotypes.
[b] Model adjusted by age and VacA genotypes.
[c] Model adjusted by age and sex. A p < 0.05 was considered statistically significant.

6.18, CI: 1.25-30.51, p = 0.025). That information is congruent with the multifactorial nature of gastric cancer and highlights the importance of analyzing variables in multivariate models that enable the confounding or interactive effect of the factors possibly involved in gastric carcinogenesis to be evaluated.

In addition, our results could be useful in the effort to predict the risk of progression to cancer. For example, it is known that only 0.8% of the patients with atrophy and 3.9% of the patients with dysplasia end up developing cancer[14,25]. However, it is difficult to try and predict which patients will fall into those percentages. Based on our results, we can suggest that patients with the s1m1 genotype are a special risk group, in whom more periodic endoscopic and histopathologic follow-up would be justified.

The pathogenic potential of the VacA/s1m1 genotype can be explained through different mechanisms, such as the capacity to induce cellular vacuolization, mitogen-activated protein kinase (MAPK) activation, and immune system inhibition[26,27]. Those mechanisms would presumably facilitate the neoplastic transformation in the gastric mucosa[28].

The number of Colombian articles on bacterial genotypes and cancer is small. Yamaoka et al. compared the VacA/s1m1 genotype distribution in four countries. Even though they showed that the s1m1 subtype predominated in Colombia, they found no statistically significant associations with the risk for cancer[29]. In contrast, Citelly et al. showed that the s1m1 genotype was present in 81% of the patients with cancer and only in about 40% of the patients with gastritis, with said difference having statistical significance[10]. In the study by Sicinschi et al., they compared the numbers of the VacA/s1m1 strains in a high-risk area and a low-risk area for gastric cancer, located in the department of Nariño (Colombia). Those authors concluded that the cytotoxic genotype was more frequent in the high-risk area (p = 0.044)[11]. Regarding the CagA genotype, a higher risk for developing cancer or dysplasia in the patients with the CagA+ genotype was not shown in our study, which was similar to the results reported by Trujillo et al.[9] but different from those reported by Citelly et al. and Sicinschi et al. Those studies had contradictory conclusions, did not calculate the magnitude of association, and were focused on populations that were culturally and geographically different from our study population. To the best of our knowledge,

the present study is the first analytic study to calculate the risk for developing dysplasia and adenocarcinoma by evaluating the genotypic variants of *H. pylori* in a population from the department of Cauca.

It is important to underline the presence of relatively wide confidence intervals (especially for the age variable) in our results that were due to the fact that only five cases of dysplasia and cancer were documented in patients under 50 years of age, and because the strict application of the inclusion criteria conditioned an important reduction in the number of study patients. Nevertheless, we believe that, given the p values, the limits of the confidence intervals, and the calculations obtained in the multivariate model, the conclusion can be made that age and the VacA s1m1 genotype are variables that parsimoniously explain the risk for developing dysplasia and cancer. To obtain accurate calculations and more precise confidence intervals, future studies should include a larger number of patients with cancer and other factors of interest (e.g., BabA genotypes, salt intake, dietary habits, among others) should be included in the multivariate modeling.

In Colombia, despite finding geographic zones with a similar prevalence of *H. pylori* infection, disparate incidence rates of cancer have been documented, a phenomenon called the ''Colombian enigma'' by certain researchers[3]. The genetic variability of *H. pylori* has been suggested as an explanation of said enigma. Even though the findings of our study appear to lend support to that theory, certain limitations must first be taken into consideration. Our study population was taken from a single geographic region. To determine whether the genetic variability of *H. pylori* has an influence on the incidence of cancer in the different geographic areas, comparative genotyping studies should be conducted in zones of high risk and low risk for cancer. The study by Trujillo et al.[9] employed such a similar methodological design but found no significant differences between bacterial genotypes in two regions of Colombia with contrasting risks for cancer. Therefore, we also suggest that the selection of patients in future studies be complemented with a genetic ancestry analysis, whenever the Colombian population chosen is genetically diverse. The studies by Kodaman et al. have demonstrated the importance of the ancestral composition of the populations and the co-evolutionary relation to bacterial infection[30].

In conclusion, it is plausible to state that male patients above 50 years of age are a group at higher risk for gastric cancer. Said risk can be potentiated by the presence of cytotoxic variants of VacA. Therefore, stricter medical follow-up is justified in patients with the VacA/s1m1 genotype. We believe this information can be used for the development of a risk index for gastric cancer, to enable opportune diagnosis of the disease and optimize the use of medical resources in the population at greater risk.

Financial disclosure

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424, http://dx.doi.org/10.3322/caac.21492.
2. Sierra MS, Cueva P, Bravo LE, et al. Stomach cancer burden in Central and South America. Cancer Epidemiol. 2016;44:S62–73, http://dx.doi.org/10.1016/j.canep.2016.03.008.
3. Correa P, Piazuelo MB. Gastric cancer: the Colombian enigma. Rev Colomb Gastroenterol. 2010;25:334–7.
4. Correa P, Camargo MC, Piazuelo MB. Overview and pathology of gastric cancer. In: Wang TC, Fox JG, Giraud AS, editors. Biol. NY: Springer New York: Gastric Cancers; 2009. p. 1–24, http://dx.doi.org/10.1007/978-0-387-69182-4_1.
5. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histoclinical classification. Acta Pathol Microbiol Scand. 1965;64:31–49, http://dx.doi.org/10.1111/apm.1965.64.1.31.
6. Wroblewski LE, Peek RM. *Helicobacter pylori* in gastric carcinogenesis: mechanisms. Gastroenterol Clin North Am. 2013;42:285–98, http://dx.doi.org/10.1016/j.gtc.2013.01.006.
7. Miehlke S, Kirsch C, Agha-Amiri K, et al. The *Helicobacter pylori* vacA s1, m1 genotype and cagA is associated with gastric carcinoma in Germany. Int J Cancer. 2000;87:322–7.
8. Castillo-Rojas G, Mazarí-Hiriart M, López-Vidal Y. *Helicobacter pylori*: focus on CagA and VacA major virulence factors. Salud Pública Méx. 2004;46:538–48, http://dx.doi.org/10.1590/s0036-36342004000600010.
9. Trujillo E, Martínez T, Bravo MM. Genotyping of *Helicobacter pylori* virulence factors vacA and cagA in individuals from two regions in Colombia with opposing risk for gastric cancer. Biomedica. 2014;34:567–73, http://dx.doi.org/10.1590/S0120-41572014000400009.
10. Cittelly DM, Huertas MG, Martínez JD, et al. Los genotipos de *Helicobacter pylori* en gastritis no atrófica difieren de los encontrados en úlcera péptica, lesiones premalignas y

cáncer gástrico en Colombia. Rev Méd Chil. 2002;130:143–51, http://dx.doi.org/10.4067/S0034-98872002000200003.
11. Sicinschi LA, Correa P, Peek RM, et al. *Helicobacter pylori* genotyping and sequencing using paraffin-embedded biopsies from residents of Colombian areas with contrasting gastric cancer risks. Helicobacter. 2008;13:135–45, http://dx.doi.org/10.1111/j.1523-5378.2008.00554.x.
12. Hassan TMM, Al-Najjar SI, Al-Zahrani IH, et al. *Helicobacter pylori* chronic gastritis updated Sydney grading in relation to endoscopic findings and *H. pylori* IgG antibody: diagnostic methods. J Microsc Ultrastruct. 2016;4:167–74, http://dx.doi.org/10.1016/j.jmau.2016.03.004.
13. Sugimoto M, Wu J-Y, Abudayyeh S, et al. Unreliability of results of PCR detection of *Helicobacter pylori* in clinical or environmental samples. J Clin Microbiol. 2009;47:738–42, http://dx.doi.org/10.1128/JCM.01563-08.
14. de Vries AC, van Grieken NCT, Looman CWN, et al. Gastric cancer risk in patients with premalignant gastric lesions: a nationwide cohort study in the Netherlands. Gastroenterology. 2008;134:945–52, http://dx.doi.org/10.1053/j.gastro.2008.01.071.
15. den Hoed CM, van Eijck BC, Capelle LG, et al. The prevalence of premalignant gastric lesions in asymptomatic patients: Predicting the future incidence of gastric cancer. Eur J Cancer. 2011;47:1211–8, http://dx.doi.org/10.1016/j.ejca.2010.12.012.
16. Ang TL, Fock KM. Clinical epidemiology of gastric cancer. Singapore Med J. 2014;55:621–8, http://dx.doi.org/10.11622/smedj.2014174.
17. Uemura N, Okamoto S, Yamamoto S, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9, http://dx.doi.org/10.1056/NEJMoa001999.
18. Nishino Y, Inoue M, Tsuji I, et al. Tobacco smoking and gastric cancer risk: an evaluation based on a systematic review of epidemiologic evidence among the Japanese population. Jpn J Clin Oncol. 2006;36:800–7, http://dx.doi.org/10.1093/jjco/hyl112.
19. Sasazuki S, Sasaki S, Tsugane S. Cigarette smoking, alcohol consumption and subsequent gastric cancer risk by subsite and histologic type. Int J Cancer. 2002;101:560–6, http://dx.doi.org/10.1002/ijc.10649.
20. Camargo MC, Goto Y, Zabaleta J, et al. Sex hormones, hormonal interventions, and gastric cancer risk: a meta-analysis. Cancer Epidemiol Biomark Prev. 2012;21:20–38, http://dx.doi.org/10.1158/1055-9965.EPI-11-0834.
21. Miftahussurur M, Yamaoka Y, Graham DY. *Helicobacter pylori* vacuolating cytotoxin and gastric cancer risk: reconsidered. Transl Cancer Res. 2016;5:S557–60, http://dx.doi.org/10.21037/tcr.2016.09.33.
22. Basso D, Zambon C-F, Letley DP, et al. Clinical relevance of *Helicobacter pylori* cagA and vacA gene polymorphisms. Gastroenterology. 2008;135:91–9, http://dx.doi.org/10.1053/j.gastro.2008.03.041.
23. González CA, Figueiredo C, Lic CB, et al. Helicobacter pylori cagA and vacA genotypes as predictors of progression of gastric preneoplastic lesions: a long-term follow-up in a high-risk area in Spain. Am J Gastroenterol. 2011;106:867–74, http://dx.doi.org/10.1038/ajg.2011.1.
24. Pormohammad A, Ghotaslou R, Leylabadlo HE, et al. Risk of gastric cancer in association with *Helicobacter pylori* different virulence factors: a systematic review and meta-analysis. Microb Pathog. 2018;118:214–9, http://dx.doi.org/10.1016/j.micpath.2018.03.004.
25. Tava F, Luinetti O, Ghigna MR, et al. Type or extension of intestinal metaplasia and immature/atypical ''indefinite-for-dysplasia'' lesions as predictors of gastric neoplasia. Hum Pathol. 2006;37:1489–97, http://dx.doi.org/10.1016/j.humpath.2006.05.012.

26. Cover TL, Blanke SR. *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. Nat Rev Microbiol. 2005;3:320–32, http://dx.doi.org/10.1038/nrmicro1095.

27. Djekic A, Müller A. The immunomodulator VacA promotes immune tolerance and persistent *Helicobacter pylori* infection through its activities on T-Cells and antigen-presenting cells. Toxins. 2016;8:187, http://dx.doi.org/10.3390/toxins8060187.

28. Bridge DR, Merrell DS. Polymorphism in the *Helicobacter pylori* CagA and VacA toxins and disease. Gut Microbes. 2013;4:101–17, http://dx.doi.org/10.4161/gmic.23797.

29. Thi Huyen Trang T, Thanh Binh T, Yamaoka Y. Relationship between vacA Types and development of gastroduodenal diseases. Toxins. 2016;8:182, http://dx.doi.org/10.3390/toxins8060182.

30. Kodaman N, Pazos A, Schneider BG, et al. Human and *Helicobacter pylori* coevolution shapes the risk of gastric disease. Proc Natl Acad Sci U S A. 2014;111:1455–60, http://dx.doi.org/10.1073/pnas.1318093111.