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Original Article

Association of *Helicobacter pylori vacA* polymorphisms with the risk of gastric precancerous lesions in a Moroccan population

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Abstract
Introduction: *Helicobacter pylori* infection is the major risk factor of atrophic gastritis and intestinal metaplasia. The *vacA* gene is one of the most virulence factors of *H. pylori* and genetic diversity in its s, m, i, and d regions is associated with gastric lesions severity. This study aimed to investigate the association of *vacA* s, m, i, and d regions with the risk of atrophic gastritis and intestinal metaplasia in a Casablanca population.
Methodology: A total of 210 patients suffering from gastric lesions (chronic gastritis, atrophic gastritis, and intestinal metaplasia) were enrolled. The type of lesion was diagnosed by histological examination. Detection of *H. pylori* infection and genotyping of *vacA* regions were carried out by PCR.
Results: The prevalence of *H. pylori* was 95%. The most common *vacA* genotypes were s2 (51.5%), m2 (77%), i2 (60.5%), and d2 (58.5%). *VacA* s1, m1, and i1 genotypes were associated with a high risk of intestinal metaplasia, while the *vacA* d1 genotype increases the risk of atrophic gastritis and intestinal metaplasia. The most common *vacA* combination was s2/m2/i2/d2 (52%), and it was more detected in chronic gastritis. The moderate virulent *vacA* combination (s1/m2/i1/d1) increases the risk of atrophic gastritis, while the most virulent *vacA* combination (s1/m1/i1/d1) increases the risk of intestinal metaplasia.
Conclusions: Genotyping of *vacA* d region might be a reliable marker for the identification of *vacA* virulent strains that represent a high risk of developing precancerous lesions (atrophic gastritis and intestinal metaplasia).

Key words: Atrophic gastritis; *Helicobacter pylori*; intestinal metaplasia; *VacA* gene.

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Introduction

Helicobacter pylori infection systematically leads to chronic gastritis (CG), which can develop into more severe pathologies such as gastric ulcer, MALT lymphoma, and gastric cancer (GC). GC is a multi-step pathology that develops through a series of lesions known as gastric carcinogenesis and includes: CG, atrophic gastritis (AG), intestinal metaplasia (IM), dysplasia, and GC [1]. The mechanisms by which AG and IM (known as precancerous lesions) develop are linked to a complex interaction between *H. pylori* virulence factors, human genetics, and environmental factors.

H. pylori is a genetically heterogeneous bacterium and genetic polymorphisms of its virulence factors affect its pathogenicity [2]. For instance, variations of the *vacuolating cytotoxin A* (*vacA*) gene have been proposed as a means of identifying virulent strains involved in the occurrence of gastric diseases [3].

The *vacA* gene is one of the most important virulence factors of *H. pylori*. This gene encodes for the multifunctional toxin VacA involved in several deleterious biological activities, such as vacuolization, apoptosis, tight junction disruption, and suppression of T cell activation [4]. The *vacA* gene is present in all *H. pylori* strains and comprises four polymorphic regions: signal (s), middle (m), intermediate (i), and deletion (d) region. Each *vacA* region is divided into two subtypes: s1, s2, m1, m2, i1, i2, d1, and d2 [5–7].

The *vacA* s region encodes for the signal peptide of the VacA protein. The *vacA* s1 genotype encodes for the whole signal peptide while the *vacA* s2 genotype encodes for a short signal peptide which results in a low vacuolating activity [8,9]. The *vacA* m region is responsible for the binding of the VacA protein to host cells. The *vacA* m1 genotype is more active and binds to a wider range of host cells than the m2 genotype [9]. The *vacA* i and d regions have been recently discovered [6,7]. In the *vacA* i region, the i1 genotype is associated with high vacuolation activity than the i2 genotype [6]. In the *vacA* d region, the d1 genotype is characterized by the absence of a 69 to 81 bp deletion, while the d2 genotype exhibits this deletion [7]. Several *vacA* combinations of these genotypes exist, and each of them is more or less associated with the risk of precancerous lesions and GC development.

GC is one of the most aggressive neoplasms and it is associated with a poor prognosis. Because of its late diagnosis, most Moroccan patients detected are at advanced stages of the disease, which results in a five-year survival rate of less than 15% [10]. Finding a marker for the early diagnosis of patients at high risk of developing this cancer is an important step in reducing its mortality. Our study aimed to investigate the polymorphisms of *vacA* s, m, i, and d regions and their association with gastric precancerous lesions in a Casablanca population, in order to use these regions as predictive markers in the identification and follow-up of patients that present high risks of developing this cancer.

Methodology

Study population

A total of 210 patients consulting in the gastroenterology service of Ibn Rochd University Hospital Center of Casablanca (Morocco) and suffering from digestive pains were included in this study. From all patients, 6 biopsies (2 from antrum, 2 from fundus, and 2 from lesser curvature) have been sampled. Three biopsies (1 from antrum, 1 from fundus, and 1 from lesser curvature) were used for histological examination and the other three biopsies were used for molecular detection. All participants were informed of their inclusion in the study and agreed to it on a writing form. The study protocol has been performed under the ethical standards of Helsinki and was approved by the ethical committee of the Pasteur Institute of Morocco.

Histology

The biopsy samples were transported in 10% formalin and embedded in paraffin. Multiple histological sections were obtained from each biopsy. Biopsy sections were then obtained and stained with hematoxylin-eosin for the detection of gastric lesions. The blades were read by a pathologist.

PCR for H. pylori detection

Total DNA was extracted from gastric biopsies using a genomic DNA extraction kit (Isolate Genomic DNA Kit, Bioline, Memphis, USA). Using primers described by Lu *et al*. [11], the *ureC* gene (296 bp) was amplified to detect *H. pylori* infection. The PCR reaction mixture was prepared with 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1 U of DNA Polymerase (MyTaq DNA Polymerase, BioLine, Memphis, USA), and 300 ng of DNA in a final volume of 20 µL. PCR thermocycling conditions for *H. pylori* detection were: 1 cycle at 95 °C for 1 minute, 35 cycles at 95° C for 15 seconds, 55 °C for 30 seconds, 72 °C for 30 seconds, and a final extension cycle at 72 °C for 7 minutes.

Genotyping of vacA regions

H. pylori-positive samples were subjected to PCR for genotyping of *vacA* s, m, i, and d regions. Primers used in this study are listed in Table 1 [5-7,12].

For *vacA* s and m regions, the PCR reactions mixtures were prepared with 0.5 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 1 U of MyTaq DNA Polymerase (MyTaq DNA Polymerase, BioLine, Memphis, USA), and 300 ng of DNA in a final volume of 20 µL.

For *vacA* i and d regions, the PCR reactions mixtures were prepared with 0.75 mM dNTPs, 2.25 mM $MgCl_2$, 0.4 µM of each primer, 1 U of MyTaq DNA Polymerase (MyTaq DNA Polymerase, BioLine,

Table 1. Primers used in this study.

Region amplified	Primer name	Primer sequence	Size (bp)	References
s (s1/s2)	VAI-F	ATGGAAATACAACAAACACAC	s1 = 259	[5]
	VAI-R	CTGCTTGAATGCGCCAAAC	s2 = 286	
m (m1/m2)	VAG-F	CAATCTGTCCAATCAAGCGAG	m1 = 567	[12]
	VAG-R	GCGTCAAAATAATTCCAAGG	m2 = 642	
i1	VACF1	GTTGGGATTGGGGGAATGCCG	426	[6]
	C1R	TTAATTTAACGCTGTTTGAAG		
i2	VACF1	GTTGGGATTGGGGGAATGCCG	432	
	C2R	GATCAACGCTCTGATTTGA		
d (d1/d2)	VAS-5F	ACTAATATTGGCACACTGGATTTG	d1 = 367-379	[7]
	VAGF-R	CTCGCTTGATTGGACAGATTG	d2 = 298	

Memphis, USA), and 300 ng of DNA in a final volume of 20 µL. All PCR thermocycling conditions for genotyping of *vacA* regions are listed in Table 2.

Statistical analysis

R software version 3.4.0 was used to conduct statistical analysis. Chi-square and ANOVA tests were performed to assess all associations between gastric lesions, age, gender, and *vacA* s, m, i, and d regions. The association between *vacA* d genotypes and *vacA* s, m, and i combinations were calculated using the Fisher test.

For the association between gastric lesions and *vacA* combinations, gastric lesions were considered as the dependent variable, and *vacA* s1/m2/i1/d1 and s1/m1/i1/d1 combinations as the predictor variables. The CG group and *vacA* s2/m2/i2/d2 combination were taken respectively as the control group and the reference strain. Results were expressed as odds ratio (OR), 95% confidence intervals (95% CI), and *p*-values.

Results

Population characteristics

The population was constituted of 99 (47%) males and 111 (53%) females. The mean age of the population was 49 ± 16. According to histological examination, 61% of patients were diagnosed with CG, 25% with AG, and 13% with IM.

Gastric lesions severity was increasing with age, but without being statistically meaningful ($p = 0.39$) (Table 3). Concerning gender, the frequency of females and males diagnosed with CG was the same (48 and 52%, respectively). AG was more diagnosed among females, whereas IM was predominant among males (Table 3). Association between gender and gastric lesions severity was statistically significant ($p = 0.04$).

The presence of *H. pylori* was detected in the gastric mucosa of 200 patients (95%): 121 (94%) cases in CG, 51(96%) cases in AG, and 28 (100%) cases in IM.

Prevalence of vacA genotypes

All *vacA* regions were determined for all the 200 *H. pylori*-positive patients. In all *vacA* regions, a dominance of the inactive form of the *vacA* genotype (s2, m2, i2, and d2) was observed (Table 4).

Association between vacA genotypes and gastric precancerous lesions

The frequency of the *vacA* s1 genotype was shown to increase with gastric lesions severity: 41% in CG, 57% in AG, and 64% in IM. The *vacA* m1 genotype was detected with low frequency in CG and AG (18 and 20%, respectively) while it was higher in IM (50%). The frequency of the *vacA* i1 genotype was found to increase with gastric lesions severity: 31% in CG, 45% in AG, and 64% in IM. Similarly, the frequency of the *vacA* d1 genotype increased with gastric lesions severity: 31% in CG, 49% in AG, and 71% in IM (Table 4). Distributions of *vacA* s, m, i, and d genotypes according to gastric lesions severity were statistically significant (Table 4).

According to table 4, the association between *vacA* s, m, and i regions with the risk of AG was not statistically significant. In contrast, the *vacA* d region was shown to increase the risk of AG by an OR of 2.1 (95% CI = 1.07 – 4.1, *p*-value = 0.02).

Table 2. PCR thermocycling conditions for genotyping of *vacA* regions used in this study.

***VacA* region amplified**	**PCR thermocycling conditions**
s region	1 cycle at 95 °C for 1 min, 35 cycles at 95 °C for 1 min, 56 °C for 50 s, 72 °C for 1 min and a final extension cycle at 72 °C for 7 min
m region	1 cycle at 95 °C for 1 min, 35 cycles at 95 °C for 1 min, 57 °C for 50 s, 72 °C for 1 min and a final extension cycle at 72 °C for 7 min
i region	1 cycle at 95 °C for 1 min, 35 cycles at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min and a final extension cycle at 72 °C for 7 min
d region	1 cycle at 95 °C for 1 min, 35 cycles at 95 °C for 1 min, 59 °C for 50 s, 72 °C for 1 min and a final extension cycle at 72 °C for 7 min

Table 3. Distribution of gastric pathologies according to age and gender.

	CG	**AG**	**IM**	***p*-value**
	n = 129 (%)	**n = 53 (%)**	**n = 28 (%)**	
Age (mean ± sd)	48 ± 17	49 ± 13	53 ± 17	0.39 *
Gender				
Males	62 (48)	19 (36)	18 (64)	0.04 **
Females	67 (52)	34 (64)	10 (36)	

CG: chronic gastritis; AG: atrophic gastritis; IM: intestinal metaplasia; sd: standard deviation. * ANOVA test; **: Chi-square test.

Table 4. Prevalence and association of vacA genotypes and gastric lesions.

	Prevalence of *vacA* genotypes n (%)	**Association of *vacA* genotypes with gastric lesions**			
		CG n = 121 (%)	**AG n = 51 (%)**	**IM n = 28 (%)**	***p*-value ***
s region					
s1	97 (48.5)	50 (41)	29 (57)	18 (64)	
s2	103 (51.5)	71 (59)	22 (43)	10 (36)	0.03
** OR, 95% CI, *p*-value		-	1.87, 0.96 – 3.62, 0.06	2.55, 1.08 – 6, 0.03	
m region					
m1	46 (23)	22 (18)	10 (20)	14 (50)	
m2	154 (77)	99 (82)	41 (80)	14 (50)	0.001
** OR, 95% CI, *p*-value		-	1.09, 0.47 – 2.52, 0.83	4.5, 1.87 – 10.77, 0.001	
i region					
i1	79 (39.5)	38 (31)	23 (45)	18 (64)	
i2	121 (60.5)	83 (69)	28 (55)	10 (29)	0.003
** OR, 95% CI, *p*-value		-	1.79, 0.91 – 3.51, 0.11	3.93, 1.65 – 9.31, 0.002	
d region					
d1	83 (41.5)	38 (31)	25 (49)	20 (71)	
d2	117 (58.5)	83 (69)	26 (51)	8 (29)	< 0.001
** OR, 95% CI, *p*-value		-	2.1, 1.07 – 4.1, 0.02	5.46, 2.2 – 13.5, < 0.001	

*All *p*-values were calculated using the Chi-square test. **Odds ratios were calculated using the twoby2 function and CG as a control group. OR: odds ratio; 95% CI: 95% confidence interval; CG: chronic gastritis; AG: atrophic gastritis; IM: intestinal metaplasia.

Table 5. Distribution of the vacA d genotypes according to vacA s, m, and i combinations.

***VacA* d region**	***VacA* combinations n (%)**					***p*-value ***
	s1/m1/i1	**s1/m2/i1**	**s1/m2/i2**	**s2/m2/i2**	**s1/m1/i2**	
d1	44 (55)	28 (35)	2 (2.5)	4 (5)	2 (2.5)	< 0.001
d2	0 (0)	4 (3.3)	12 (10)	104 (86.7)	0 (0)	

*The *p*-value was calculated using Fisher test.

Table 6. Prevalence and distribution of *vacA* combinations according to gastric pathologies.

	Prevalence of *vacA* combinations n (%)	**Distribution of *vacA* combinations according to gastric pathologies**		
		CG n = 121 (%)	**AG n = 51 (%)**	**IM n = 28 (%)**
s2/m2/i2/d2	104 (52)	71 (59)	25 (49)	8 (29)
s1/m1/i1/d1	44 (22)	22 (18)	8 (16)	14 (50)
s1/m2/i1/d1	28 (14)	12 (10)	12 (23)	4 (14)
s1/m2/i2/d2	12 (6)	8 (6.6)	4 (8)	-
s2/m2/i2/d1	4 (2)	2 (1.6)	-	2 (7)
s1/m2/i1/d2	4 (2)	4 (3)	-	-
s1/m1/i2/d1	2 (1)	-	2 (4)	-
s1/m2/i2/d1	2 (1)	2 (1.6)	-	-

CG: chronic gastritis; AG: atrophic gastritis; IM: intestinal metaplasia.

Table 7. Association between vacA s1/m2/i1/d1 combination and the risk of precancerous lesions.

	s2/m2/i2/d2 n (%)	**s1/m2/i1/d1 n (%)**	**OR ***	**95% CI**	***p*-value**
CG	71 (86)	12 (14)	-	-	-
AG	25 (68)	12 (32)	2.84	1.13 – 7.13	0.02
IM	8 (67)	4 (33)	2.95	0.76 – 11.37	0.1

*Odds ratios were calculated using the twoby2 function, and CG and *vacA* s2/m2/i2/d2 combination as a control group and reference strain, respectively. CG: chronic gastritis; AG: atrophic gastritis; IM: intestinal metaplasia; OR: odds ratio; 95% CI: 95% confidence interval.

In the case of IM, the *vacA* s1, m1, i1, and d1 genotypes were found to increase the risk of IM with an OR of 2.55 (95% CI = 1.08 – 6, *p*-value = 0.03), 4.5 (95% CI = 1.87 – 10.77, *p*-value = 0.001), 3.93 (95% CI = 1.65 – 9.31, *p*-value = 0.003), and 5.46 (95% CI = 2.2 – 13.5, *p*-value < 0.001), respectively (Table 4).

Association between vacA d genotypes and vacA combinations

Our results showed that the frequency of *vacA* d1 genotype was elevated in the *vacA* s1/m1/i1 combination (55%), followed by the *vacA* s1/m2/i1 combination (35%), the *vacA* s2/m2/i2 combination (5%), the *vacA* s1/m2/i2 combination (2.5%), and the *vacA* s1/m1/i2 combination (2.5%). In contrast, the *vacA* d2 genotype was more detected in the *vacA* s2/m2/i2 combination (86.7%), followed by the *vacA* s1/m2/i2 combination (10%), and the *vacA* s1/m2/i1 combination (3.3%) (Table 5).

Prevalence of vacA combinations

Considering all *vacA* regions together, the *vacA* combinations observed in this study are listed in Table 6. Our population was characterized by a dominance of the *vacA* s2/m2/i2/d2 combination (52%), followed by the *vacA* s1/m1/i1/d1 and s1/m2/i1/d1 combinations (22 and 14%, respectively).

Distribution of vacA combinations according to gastric lesions

In CG, the most common *vacA* combination was s2/m2/i2/d2 (59%), followed by the *vacA* s1/m1/i1/d1 combination (18%), the *vacA* s1/m2/i1/d1 combination (10%), the *vacA* s1/m2/i2/d2 combination (6.6%), the *vacA* s1/m2/i1/d2 combination (3%), the *vacA* s2/m2/i2/d1 and s1/m2/i2/d1 combinations (1.6%).

In the case of AG, the most common *vacA* combination was s2/m2/i2/d2 (49%), followed by the *vacA* s1/m2/i1/d1 combination (23%), the *vacA* s1/m1/i1/d1 combination (16%), the *vacA* s1/m2/i2/d2 combination (8%), and the *vacA* s1/m1/i2/d1 combination (4%).

In IM, the most common *vacA* combination was s1/m1/i1/d1 (50%), followed by s2/m2/i2/d2 and s1/m2/i1/d1 combinations (29 and 14%, respectively). The *vacA* s2/m2/i2/d1 combination was detected in 7%, while the other *vacA* combinations were totally absent.

Table 6 shows that the frequency of the lowest virulent *vacA* combination, s2/m2/i2/d2, decreases according to gastric lesions severity: 59% in CG, 49% in AG, and 29% in IM. In the case of the *vacA* s1/m2/i1/d1 combination, considered as a moderate virulent combination, it was more detected in AG (23%) compared to CG and IM (10 and 14%, respectively). In contrast, the frequency of the most virulent *vacA* combination, s1/m1/i1/d1, increases according to gastric lesions severity: 18% in CG, 16% in AG, and 50% in IM.

Association between vacA combinations and the risk of gastric precancerous lesions

By taking CG as a control group and *vacA* s2/m2/i2/d2 combination as a reference strain, the risks of developing AG and IM following infection with the *vacA* s1/m1/i1/d1 and s1/m2/i1/d1 combinations were estimated.

According to Table 7, the frequency of *vacA* s1/m2/i1/d1 combination was higher in AG (32%) compared to CG (14%). In contrast, the frequency of *vacA* s2/m2/i2/d2 combination was lower in AG (68%) compared to CG (86%). Therefore, the risk of developing AG lesion in patients carrying the *vacA* s1/m2/i1/d1 strains was higher with a factor of OR = 2.84 (95% CI = 1.13 – 7.13, *p*-value = 0.02), compared to those carrying the *vacA* s2/m2/i2/d2 strains.

The frequency of the *vacA* s1/m2/i1/d1 combination was higher among patients suffering from IM (33%) compared to CG (14%), while the frequency of the *vacA* s2/m2/i2/d2 combination was lower in IM (67%) compared to CG (86%). However, no association was found between the risk of developing IM lesion and patients carrying the *vacA* s1/m2/i1/d1 combination (OR = 2.95, 95% CI = 0.76 – 11.37, *p*-value = 0.1).

According to Table 8, the distribution of *vacA* s1/m1/i1/d1 and s2/m2/i2/d2 combinations were the same in AG and CG. Thus, no association was found between the *vacA* s1/m1/i1/d1 combination and the risk of AG (OR = 1.03, 95% CI = 0.4 – 2.61, *p*-value = 1).

Table 8. Association between vacA s1/m1/i1/d1 combination and the risk of precancerous lesions.

	s2/m2/i2/d2 n (%)	s1/m1/i1/d1 n (%)	OR*	95% CI	*p*-value
CG	71 (76)	22 (24)	-	-	-
AG	25 (76)	8 (24)	1.03	0.4 – 2.61	1
IM	8 (36)	14 (64)	5.64	2.09 – 15.22	< 0.001

*Odds ratios were calculated using the twiby2 function, and CG and *vacA* s2/m2/i2/d2 combination as a control group and reference strain, respectively. CG: chronic gastritis; AG: atrophic gastritis; IM: intestinal metaplasia; OR: odds ratio, 95% CI: 95% confidence interval.

In IM, the frequency of the *vacA* s1/m1/i1/d1 combination was higher (64%) compared to CG (24%), while the frequency of the *vacA* s2/m2/i2/d2 combination was lower in IM (36%) compared to CG (76%). Therefore, a significant association was found between the risk of developing IM lesion in patients carrying the *vacA* s1/m1/i1/d1 strains (OR = 5.64, 95% CI = 2.09 – 15.22, *p*-value < 0.001).

Discussion

Since the discovery of the *vacA* s, m, and i regions, numerous studies have investigated their association with the risk of precancerous lesions. However, the recently discovered *vacA* d region remains poorly studied. In this study, we characterized the polymorphisms of the *vacA* s, m, i, and d regions in order to study their association with the development of precancerous lesions.

In *vacA* s, m, and i regions, the most common genotypes were *vacA* s2 (51.5%), m2 (77%), and i2 (60.5%). This observation is similar to the epidemiological studies conducted on Moroccan, Tunisian, Egyptian, and Kenyan populations [13–16], but differs from a Senegalese study, where *vacA* s1, m1, and i1 genotypes were predominant [17].

Concerning the *vacA* d region, the majority of *H. pylori* strains were *vacA* d2 genotype (58.5%). Such finding has been reported by several Iranian studies [18–21], while other studies revealed a dominance of the *vacA* d1 genotype [22,23]. In Africa, there is no data regarding the prevalence of *vacA* d region, so further studies are needed to establish an accurate profile of this region.

The distribution of *vacA* genotypes among gastric lesions showed that the frequency of *vacA* s1, m1, and i1 genotypes tends to increase in AG compared to CG, but without reaching a statistically significant association (Table 4). In IM, all active forms of the *vacA* regions (s1, m1, and i1 genotypes) were found to be associated with the development of this lesion. Association between *vacA* genotypes and the development of gastric lesions varied among epidemiological studies. Some reports found that *vacA* s1, m1, and i1 genotypes increased the risk of both AG and IM [24,25], while others found that *vacA* s1, m1, and i1 genotypes were only associated with the risk of IM [14,26,27].

In the case of the *vacA* d region, the *vacA* d1 genotype was found to be associated with AG and IM. Ogiwara et al reported a positive association between *vacA* d1 genotype and the development of AG [7]. In addition, the *vacA* d1 genotype was found to increase the risk of GC by numerous studies [18–20,23]. However, no study has assessed the association between the *vacA* d region and IM.

The combination of the *vacA* s, m, and i genotypes allows the differentiation of the vacuolating activity of the VacA protein between *H. pylori* strains. It is known that the *vacA* s1/m1/i1 combinations induce cell vacuolation while the *vacA* s2/m2/i2 combinations do not. In the case of the *vacA* s1/m2 combinations, the presence of the i1 genotype is associated with a cellular vacuolation activity, while the presence of the i2 genotype is associated with the absence of the vacuolation activity [3,6,25].

In our population, most of our *vacA* d1 genotype cases were detected in the active forms of v*acA* combinations (s1/m1/i1 and s1/m2/i1). This observation is similar to previous studies [7,18,22]. In contrast, the inactive forms of *vacA* combinations (s1/m2/i2 and s2/m2/i2) were characterized by a predominance of the *vacA* d2 genotype. Even though the physiological role of the *vacA* d region remains undiscovered, it seems that the *vacA* d1 and d2 genotypes are highly associated with the active and inactive forms of *vacA* combinations, which are respectively characterized by high and low vacuolation activity.

The mosaic combination of the *vacA* s, m, i, and d regions can lead to several *vacA* combinations. Our population is characterized by the predominance of the nonvirulent *vacA* combination, s2/m2/i2/d2, followed by the most virulent *vacA* combination, s1/m1/i1/d1. In an Algerian study, the *vacA* s2/m2/i2/d2 was the most common combination [28]. Several African studies (Tunisia, Morocco, and Egypt) have also shown the predominance of the *vacA* s2/m2 combination in their population [13,15,29]. Moreover, a Moroccan and Kenyan study found a high prevalence of the *vacA* s2/m2/i2 combination, followed by the *vacA* s1/m1/i1 combination [14,16]. In contrast, the *vacA* s1/m1/i1 combination was predominantly detected in a Senegalese study [17].

Our finding demonstrated the association between the *vacA* d2 genotype with the least virulent *vacA* combination (s2/m2/i2). In addition, most African *H. pylori* strains are characterized by the predominance of the *vacA* s2/m2 and s2/m2/i2 combinations. Based on these observations, we might suggest that the African *vacA* genetic profile could belong to the s2/m2/i2/d2 combination. However, more studies are needed to confirm this hypothesis.

The results of our study suggest that patients infected with the *vacA* s1/m1/i1/d1 combination are more susceptible to develop IM (OR = 5.64, 95% CI = 2.09 – 15.22, *p*-value < 0.001) than AG (OR = 1.03, 95% CI = 0.4 – 2.61, *p*-value = 1). Winter et al showed that the *vacA* s1/i1 combinations are associated with a high risk of IM compared to the *vacA* s2/i2 combinations [26]. Moreover, a follow-up study conducted by Gonzalez et al showed that progression toward IM was more frequent in patients infected with the *vacA* s1/m1 combination than patients infected with the *vacA* s2/m2 combination [30].

The rapid evolution from a simple CG to more severe lesions is linked to the type of *vacA* combination. Indeed, *H. pylori* strains carrying the *vacA* s1/m1/i1 combination are known to be more virulent than *H. pylori* strains carrying the *vacA* s2/m2/i2 combination. It was shown that *vacA* s2/m2/i2 combinations do not cause vacuolation on epithelial cells, while *vacA* s1/m1/i1 combinations are characterized by a high degree of vacuolating activity [25]. In addition, the *vacA* s1/m1/i1 combinations are highly apoptotic and induce more intense inflammation [31]. Moreover, *H. pylori* strains possessing the *vacA* s1/m1/i1 combination are more likely to carry the *cagA* gene, which is another *H. pylori* virulence factor, and considered as an oncoprotein [32–34]. All these factors can explain the association between the *vacA* s1/m1/i1/d1 combination and the high risk of IM.

In our study, the moderate virulent *vacA* s1/m2/i1/d1 combination was associated with the risk of AG (OR = 2.84, 95% CI = 1.13 – 7.13, *p*-value = 0.02). The *vacA* s1/m2/i1 combination is known to possess an intermediate vacuolating activity compared to *vacA* s1/m1/i1 combination [35]. This difference is explained by the variation encountered in the *vacA* m region, which influences host cell tropism between different *vacA* strains [36–38]. Indeed, *vacA* combinations with the m1 genotype can bind to a wider range of host cells than the *vacA* m2 genotype, which results in a great vacuolization effect [9]. This variation in cell tropism may explain the association between the *vacA* s1/m2/i1/d1 combination and the risk of AG.

Conclusions

We showed in this study that the *vacA* s2/m2/i2/d2 combination predominates in our Casablanca population. Compared to other *vacA* s, m, and i regions, the recently discovered *vacA* d region seems to be a better marker for the risk of AG and IM. In addition, the active form of the *vacA* d region was exclusively associated with the most virulent *vacA* combinations (s1/m1/i1 and s1/m2/i1). Moreover, patients infected with the *vacA* s1/m2/i1/d1 are more susceptible to develop AG, while those infected with the *vacA* s1/m1/i1/d1 combination are at high risk of developing IM. Taking together, our results show that the *vacA* d region appears to be a reliable marker for the identification of virulent *vacA* strains that are a risk factor for AG and IM development.

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Authors' contributions

Mohamed Reda Jouimyi and Fatima Maachi designed the study. Mohamed Reda Jouimyi carried out the study and wrote the manuscript. Wafaa Badre provided the biopsies. Hakima Benomar performed the histological examination. Ghizlane Bounder, Imane Essaidi, Hasna Boura, Khalid Zerouali, Halima Lebrazi, and Anass Kettani revised the manuscript. All authors read and approved the final version of the manuscript.

References

1. Correa P (1988) Chronic gastritis: a clinico-pathological classification. Am J Gastroenterol 83: 504–509.
2. Suerbaum S, Josenhans C (2007) *Helicobacter pylori* evolution and phenotypic diversification in a changing host. Nat Rev Microbiol 5: 441–452.
3. Ferreira RM, Machado JC, Figueiredo C (2014) Clinical relevance of *Helicobacter pylori vacA* and *cagA* genotypes in gastric carcinoma. Best Pract Res Clin Gastroenterol 28: 1003–1015.
4. Nejati S, Karkhah A, Darvish H, Validi M, Ebrahimpour S, Nouri HR (2018) Influence of *Helicobacter pylori* virulence factors CagA and VacA on pathogenesis of gastrointestinal disorders. Microb Pathog 117: 43–48.
5. Atherton JC, Cao P, Peek RM, Tummuru MK, Blaser MJ, Cover TL (1995) Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific vacA types with cytotoxin production and peptic ulceration. J Biol Chem 270: 17771–17777.
6. Rhead JL, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC (2007) A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. Gastroenterology 133: 926–936.
7. Ogiwara H, Sugimoto M, Ohno T, Vilaichone R-K, Mahachai V, Graham DY, Yamaoka Y (2009) Role of deletion located between the intermediate and middle regions of the *Helicobacter pylori* vacA gene in cases of gastroduodenal diseases. J Clin Microbiol 47: 3493–3500.

8. McClain MS, Cao P, Iwamoto H, Vinion-Dubiel AD, Szabo G, Shao Z, Cover TL (2001) A 12-amino-acid segment, present in type s2 but not type s1 *Helicobacter pylori* VacA proteins, abolishes cytotoxin activity and alters membrane channel formation. J Bacteriol 183: 6499–6508.
9. Torres VJ, Ivie SE, McClain MS, Cover TL (2005) Functional properties of the p33 and p55 domains of the Helicobacter pylori vacuolating cytotoxin. J Biol Chem 280: 21107–21114.
10. Elmajjaoui S, Ismaili N, Zaidi H, Elkacemi H, Hassouni K, Kebdani T, Benjaafar N (2014) epidemiological, clinical, pathological and therapeutic aspects. Clin Cancer Investig J 3: 3-8.
11. Lu JJ, Perng CL, Shyu RY, Chen CH, Lou Q, Chong SK, Lee CH (1999) Comparison of five PCR methods for detection of *Helicobacter pylori* DNA in gastric tissues. J Clin Microbiol 37: 772–774.
12. Atherton JC, Cover TL, Twells RJ, Morales MR, Hawkey CJ, Blaser MJ (1999) Simple and accurate PCR-based system for typing vacuolating cytotoxin alleles of *Helicobacter pylori*. J Clin Microbiol 37: 2979–2982.
13. Ben Mansour K, Fendri C, Zribi M, Masmoudi A, Labbene M, Fillali A, Ben Mami N, Najjar T, Meherzi A, Sfar T, Burucoa C (2010) Prevalence of *Helicobacter pylori* vacA, cagA, iceA and oipA genotypes in Tunisian patients. Ann Clin Microbiol Antimicrob 9: 10.
14. M'itonga LG, Kimang'a AN, Ngugi CW, Mutie TM (2015) Association of *Helicobacter pylori* VacA gene polymorphisms and CagA gene with clinical outcome in dyspeptic patients. Int J Health Sci Res 5: 436–444.
15. El-Khlousy M, Rahman EA, Mostafa S, Bassam A, Elgawad HA, Elnasr MS, Mohey M, Ghaith D (2016) Study of the clinical relevance of *Helicobacter pylori* virulence genes to gastric diseases among Egyptian patients. Arab J Gastroenterol 17: 90–94.
16. El Khadir M, Alaoui Boukhris S, Benajah D-A, El Rhazi K, Ibrahimi SA, El Abkari M, Harmouch T, Nejjari C, Mahmoud M, Benlemlih M, Bennani B (2017) VacA and CagA status as biomarker of two opposite end outcomes of *Helicobacter pylori* infection (gastric cancer and duodenal ulcer) in a Moroccan population. PloS One 12: e0170616.
17. Breurec S, Michel R, Seck A, Brisse S, Côme D, Dieye FB, Garin B, Huerre M, Mbengue M, Fall C, Sgouras DN, Thiberge JM, Dia D, Raymond J (2012) Clinical relevance of cagA and vacA gene polymorphisms in *Helicobacter pylori* isolates from Senegalese patients. Clin Microbiol Infect 18: 153–159.
18. Latifi-Navid S, Mohammadi S, Maleki P, Zahri S, Yazdanbod A, Siavoshi F, Massarrat S (2013) *Helicobacter pylori* vacA d1/-i1 genotypes and geographic differentiation between high and low incidence areas of gastric cancer in Iran. Arch Iran Med 16: 330–337.
19. Basiri Z, Safaralizadeh R, Bonyadi MJ, Somi MH, Mahdavi M, Latifi-Navid S (2014) *Helicobacter pylori* vacA d1 genotype predicts risk of gastric adenocarcinoma and peptic ulcers in northwestern Iran. Asian Pac J Cancer Prev 15: 1575–1579.
20. Bakhti SZ, Latifi-Navid S, Mohammadi S, Zahri S, Bakhti FS, Feizi F, Yazdanbod A, Siavoshi F (2016) Relevance of *Helicobacter pylori* vacA 3'-end region polymorphism to gastric cancer. Helicobacter 21: 305–316.
21. Basiri Z, Safaralizadeh R, Bonyadi M, Abdolmohammadi R (2017) Identification of gastric cancer-related strains of *Helicobacter pylori*: findings from single biopsy specimens for PCR and *Campylobacter*-like organism test. Jundishapur J Microbiol 10: e37006.
22. Karlsson A, Ryberg A, Dehnoei MN, Borch K, Monstein H-J (2012) Association between cagA and vacA genotypes and pathogenesis in a *Helicobacter pylori* infected population from South-eastern Sweden. BMC Microbiol 12: 129.
23. Abdi E, Latifi-Navid S, Zahri S, Yazdanbod A, Safaralizadeh R (2017) *Helicobacter pylori* genotypes determine risk of non-cardia gastric cancer and intestinal- or diffuse-type GC in Ardabil: A very high-risk area in Northwestern Iran. Microb Pathog 107: 287–292.
24. Nogueira C, Figueiredo C, Carneiro F, Gomes AT, Barreira R, Figueira P, Salgado C, Belo L, Peixoto A, Bravo JC, Bravo LE, Realpe JL, Plaisier AP, Quint WG, Ruiz B, Correa P, van Doorn LJ (2001) *Helicobacter pylori* genotypes may determine gastric histopathology. Am J Pathol 158: 647–654.
25. Ferreira RM, Figueiredo C, Bonet C, Pardo ML, Liso JMR, Alonso P, Sala N, Capella G, Sanz-Anquela JM, González CA (2012) *Helicobacter pylori* vacA intermediate region genotyping and progression of gastric preneoplastic lesions. Am J Gastroenterol 107: 145–146.
26. Winter JA, Letley DP, Cook KW, Rhead JL, Zaitoun AAM, Ingram RJM, Amilon KR, Croxall NJ, Kaye PV, Robinson K, Atherton JC (2014) A role for the vacuolating cytotoxin, VacA, in colonization and *Helicobacter pylori*-induced metaplasia in the stomach. J Infect Dis 210: 954–963.
27. Abdi E, Latifi-Navid S, Latifi-Navid H, Safarnejad B (2016) *Helicobacter pylori* vacuolating cytotoxin genotypes and preneoplastic lesions or gastric cancer risk: a meta-analysis. J Gastroenterol Hepatol 31: 734–744.
28. Raaf N, ma, Amhis W, Baiod-Chorfi S, Benhassine F, Ouar-Korichi M (2017) *Helicobacter pylori* in children: molecular characterization, antibiotic resistance and MLST typing of isolated strains in an Algerian hospital. Infect Epidemiol Microbiol 3: 73–77.
29. Alaoui Boukhris S, Boukhris SA, Benajah D-a, El Rhazi K, Ibrahimi SA, Nejjari C, Amarti A, Mahmoud M, El Abkari M, Souleimani A, Bennani B (2012) Prevalence and distribution of *Helicobacter pylori* cagA and vacA genotypes in the Moroccan population with gastric disease. Eur J Clin Microbiol Infect Dis 31: 1775–1781. doi: 10.1007/s10096-011-1501-x.
30. González CA, Figueiredo C, Lic CB, Ferreira RM, Pardo ML, Ruiz Liso JM, Alonso P, Sala N, Capella G, Sanz-Anquela JM (2011) *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 106: 867–874.
31. Foegeding NJ, Caston RR, McClain MS, Ohi MD, Cover TL (2016) An overview of *Helicobacter pylori* VacA toxin biology. Toxins (Basel) 8: 173.
32. Zambon C-F, Navaglia F, Basso D, Rugge M, Plebani M (2003) *Helicobacter pylori* babA2, cagA, and s1 vacA genes work synergistically in causing intestinal metaplasia. J Clin Pathol 56: 287–291.
33. Sugimoto M, Furuta T, Yamaoka Y (2009) Influence of inflammatory cytokine polymorphisms on eradication rates of *Helicobacter pylori*. J Gastroenterol Hepatol 24: 1725–1732.
34. Panayotopoulou EG, Sgouras DN, Papadakos KS, Petraki K, Breurec S, Michopoulos S, Mantzaris G, Papatheodoridis G, Mentis A, Archimandritis A (2010) CagA and VacA polymorphisms are associated with distinct pathological features in *Helicobacter pylori*-infected adults with peptic ulcer and non-peptic ulcer disease. J Clin Microbiol 48: 2237–2239.

35. Chang WL, Yeh YC, Sheu BS (2018) The impacts of *H. pylori* virulence factors on the development of gastroduodenal diseases. J Biomed Sci 25: 68.
36. Pagliaccia C, de Bernard M, Lupetti P, Ji X, Burroni D, Cover TL, Papini E, Rappuoli R, Telford JL, Reyrat JM (1998) The m2 form of the *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. Proc Natl Acad Sci USA 95: 10212–10217.
37. Ji X, Fernandez T, Burroni D, Pagliaccia C, Atherton JC, Reyrat JM, Rappuoli R, Telford JL (2000) Cell specificity of *Helicobacter pylori* cytotoxin is determined by a short region in the polymorphic midregion. Infect Immun 68: 3754–3757.
38. Wang WC, Wang HJ, Kuo CH (2001) Two distinctive cell binding patterns by vacuolating toxin fused with glutathione S-transferase: one high-affinity m1-specific binding and the other lower-affinity binding for variant m forms. Biochemistry 40: 11887–11896.

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