Helicobacter pylori genotypes in Lithuanian patients with chronic gastritis and duodenal ulcer

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Key words: *Helicobacter pylori; duodenal ulcer; chronic gastritis; virulence factors; cagPAI; vacA; iceA.*

Summary. *Objective. Clinical outcome of Helicobacter pylori (H. pylori) infection might be associated with specific virulence-associated bacterial genotypes. The distribution of different bacterial genotypes varies geographically. The aim of this study was to assess the relationship between cagPAI, vacA, and iceA status and severity of the disease in patients from Lithuania, infected by H. pylori.*

Material and methods. H. pylori from 81 patients (37 with duodenal ulcer and 44 with chronic gastritis) was isolated from gastric biopsy specimens and cultured. Bacterial genotypes cagPAI, vacA (s and m subtypes) and iceA were analyzed by polymerase chain reaction using specific primers.

Results. The cagPAI was identified in 59.3% of Lithuanian H. pylori strains investigated. H. pylori strains cultured from duodenal ulcer (DU) patients more frequently ($P<0.01$) contained cagPAI and vacA s1 genotypes (75.7% and 75.7%, respectively) in comparison to isolates from chronic gastritis (CG) patients (45.5% and 40.9%, respectively). Evaluation of nucleotide sequence of the vacA middle-region revealed that vacA s2/m2 genotype was more frequent in CG than in DU patients (56.8% and 24.3%, respectively; $P<0.05$). We have not found any differences in the frequency of iceA1 genotype between the DU and CG patients (46.0% and 40.9%, respectively; $P>0.05$).

Conclusion. Our study suggests that cagPAI and vacA s1 genotypes are associated with peptic ulceration in Lithuanian patients infected by H. pylori.

Introduction

Helicobacter pylori (H. pylori) infection is one of the most prevalent human bacterial infections and is associated with different gastroduodenal diseases, such as gastritis, peptic ulcer, and is an important risk factor for the development of gastric cancer and gastric lymphoma (1–4). Several potential markers of pathogenicity have been described in *H. pylori*, and some of them seem to be associated with more severe clinical outcomes of the infection (5–7). The genetic variability of *H. pylori* is high, (5, 8–10), and several genes have been identified that may play a role in the pathogenicity (*cagA, vacA, iceA*). Besides being associated with specific diseases, certain genotypes are more frequently found in certain ethnicities or geographic regions of the world (11–13). In Western populations, *H. pylori* containing cytotoxin-associated gene (*cagA*), which is a marker for a genomic pathogenecity island (*cag*PAI), is more strongly associated with more severe disease than strains lacking *cagA* (14). In contrast, nearly all East Asian strains carry the *cag*PAI independent of disease status (15, 16). Similar studies are still scanty in East European region, where prevalence of *H. pylori* infection is high, and both peptic ulcer disease and gastric adenocarcinoma are very common (17–21). In Lithuania up till now, the genetic characteristics of *H. pylori* have not been studied yet, except the pilot study by Čalkauskas *et al.* (22). The present study aimed to analyze *cag*PAI, *vacA*, and *iceA* status directly in gastric biopsy specimens from 81 patients from Lithuania in relation to clinical data.

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Materials and methods

H. pylori strains were obtained from 81 patients (39 males and 42 females; mean age, 50.04±16.35 years; range, 16–88 years) who were referred for upper gastrointestinal endoscopy at Kaunas University of Medicine Hospital due to dyspeptic symptoms. Thirty-seven patients had duodenal ulcer (DU), and forty-one – chronic gastritis (CG). Patients using aspirin or nonsteroidal anti-inflammatory drugs were excluded from the study, and none of the (CG) patients had the history of peptic ulcer disease.

Helicobacter pylori culture

H. pylori was isolated from gastric biopsy specimens and cultured on the surfaces of brain-heart infusion (BHI) agar plates supplemented with 10% horse blood, 0.4% IsoVitaleX, amphotericin B (8 μg/mL), trimethoprim (5 μg/mL), vancomycin (6 μg/mL) and Wilkins Chalgren Anaerobic Agar with 10% horse blood and Dent supplement (Oxoid). The media were incubated under microaerophilic conditions generated by CampyPak-Plus (Becton Dickinson) at 37°C from 3 to 7 days. The identity of the colonies was confirmed by typical Gram's staining and biochemical testing for urease, catalase, and oxidase. Bacterial stocks were maintained at –70°C in Brucella broth (Difco), supplemented with 15–20% glycerol.

DNA assay

Chromosomal DNA was isolated from confluent plate cultures using the QIAamp tissue kit (Qiagen, Chatsworth, Calif.). Specific PCR was generally carried out in 20-μL volumes containing 10 ng of DNA, 1 U of *Tag* polymerase (Promega, Madison, Wis.), 10 pmol of each primer per reaction, 2 to 3 mM $MgCl_2$, and 0.25 mM of each deoxynucleoside triphosphate in a standard buffer. Cycling conditions were usually 30 cycles at 94°C for 30 s, 52°C for 30 s, and 72°C for a time dependent on the expected product size (1 min per kb). The PCR primers used in this study are listed in Table 1.

Statistical analysis

Chi-square test or Fisher exact test was used for

Table 1. **Oligonucleotide primers used for polymerase chain reaction**

Genes	Primers	Sequence
*cag*PAI right junction	CagF4584 CagR5280	5′-GTTAATACAAAAGGTGGTTTCCAAAAATC[1] 5′-GGTTGCACGCATTTTCCCTTAATC[2]
*cag*PAI type I and IV	CagF4856 CagR5280	5′-GCGATGAGAAGAATATCTTTAGCG[3] 5′-GGTTGCACGCATTTTCCCTTAATC[2]
*cag*PAI type II	IS606-1692 CagR5280	5′-CTAACAATTTGCCATTATGCTGT[4] 5′-GGTTGCACGCATTTTCCCTTAATC[2]
*cag*PAI type III	Fcn unk CagR5280	5′-TGGATTAAATCTTAATGAATTATCG[5] 5′-GGTTGCACGCATTTTCCCTTAATC[2]
*cag*PAI "empty" site	Luni3 CagR5280	5′-ATAGCGTTTTGTGCATAGAATTGCGC 5′-GGTTGCACGCATTTTCCCTTAATC
vacAs1 (259 bp) or *s2* (286 bp)	VA1-F VA1-R	5′-ATGGAAATACAACAAACACAC 5′-CTGCTTGAATGCGCCAAAC
vacA s1a	SS1-F VA1-R	5′-GTCAGCATCACACCGCAAC 5′-CTGCTTGAATGCGCCAAAC
vacA s1b	SS3-F VA1-R	5′-AGCGCCATACCGCAAGAG 5′-CTGCTTGAATGCGCCAAAC
vacA m1	VA3-F VA3-R	5′-GGTCAAAATGCGGTCATGG 5′-CCATTGGTACCTGTAGAAAC
vacA m2	VA4-F VA4-R	5′-GGAGCCCCAGGAAACATTG 5′-CATAACTAGCGCCTTGCAC
iceA1	IceAF5 IceAR4	5′-GTGTTTTTAACCAAAGTATC 5′-CTATAGCCACTCCTTTGCA
iceA2	IceAF6 IceAR5	5′-GTTGGGTATATCACAATTTAT 5′-TTACCCTATTTTCTAGTAGGT

statistical analysis of the results, and significance was accepted at P<0.05.

Results

The *cag*PAI was identified in 59.3% (48/81) of Lithuanian *H. pylori* strains investigated. Thirty-three cultures, from which no *cag*PAI-specific PCR product was obtained, yielded an empty-site product of the expected 550-bp size, indicating that they truly lacked the *cag*PAI. The *vacA* gene was identified in all of the 81 *H. pylori* strains. Forty-six of the 81 (56.8%) cultures yielded a 259-bp fragment, indicating *vacA s1* alleles, and 35 had a 286-bp fragment, representing *vacA s2* alleles. None of the strains yielded a PCR product of any other size. Analysis of the 46 strains with the *s1* genotype revealed that 44 (95.7%) of them belonged to *vacA s1a* and only 2 (4%) to *vacA s1b* subtypes. The presence of *cag*PAI showed a strong association with the presence of the *vacA s1* allele (Table 2). Investigation of the alleles in the middle region of *vacA* identified 11 (13.6%) of the 81 *H. pylori* isolates as *m1* type and 54 (66.7%) as *m2* type. Alleles in the middle region of 16 strains were nontypable (*m?* type). The *vacA s1/m1* combination was found in 11 (23.9%); *s1/m2* in 20 (43.5%); and *s2/m2* was present in 34 (97.1%) of typable strains. The middle-region type *m1* was significantly (P<0.01) more frequent in *H. pylori* strains with genotype *s1,* while *m2* alleles in most cases were detected in *vacA s2* strains (Table 3). The *iceA1* genotype was found in 35 (43.2%) of the 81 Lithuanian strains, and *iceA2* genotype was detected in 40 (49.4%) of the cultures. Specific *iceA* subtypes were not revealed in 6 (7.4%) *H. pylori* strains tested by PCR.

While estimating relationship between potentially virulent *H. pylori* strains and clinical outcomes, significant differences (P<0.01) were found between isolates from DU and CG patients (Table 4). *H. pylori* strains cultured from DU patients more frequently (P<0.01) contained *cag*PAI and v*acA s1* genotype (75.7% and 75.7%, respectively) in comparison to isolates from CG patients (45.5% and 40.9%, respectively). Evaluation of nucleotide sequence of the *vacA* middle-region revealed that *vacA s2/m2* subtype was more frequent in CG than in DU patients (56.8% and 24.3%, respectively; P<0.05). We have not found any differences in the frequency of *iceA1* genotype between the groups (Table 4).

Discussion

The clinical relevance of putative virulence-associated genes of *H. pylori* is still a matter of controversy. The present study provides data on distribution of *H. pylori cag*PAI status, *vacA* and *iceA* genotypes in Lithuanian patients with confirmed diagnoses of DU and CG. One-half to two-thirds of European and U.S. *H. pylori* strains carry the *cag* pathogenicity island, a 40-kb DNA segment; many of whose genes seem to help induce interleukin-8 secretion and, thereby, a strong and potentially damaging inflammatory

Table 2. **Distribution of *cag*PAI and *vacA s1/s2* genes in 81 *Helicobacter pylori* isolates from Lithuania**

vacA subtype	*cag*PAI *type*		P
	*cag*PAI$^+$	*cag*PAI$^-$	
s1	93.8% (45/48)	3% (1/33)	<0.001
s2	6.2% (3/48)	97% (32/33)	<0.001

Table 3. **Relationship between signal sequence (*s1/s2*) and middle-region typing of *vacA* gene for 81 *Helicobacter pylori* isolates from Lithuania**

Signal sequence type	Middle-region type		
	m1	*m2*	*m?*
s1	23.9% (11/46)	43.5% (20/46)	32.6% (15/46)
s2	0% (0/35)	97.1% (34/35)	2.9% (1/35)
P	<0.01	<0.001	<0.01

Table 4. **Prevalence of *cag*PAI$^+$, *vacA*, and *iceA* genes in *Helicobacter pylori* strains isolated from Lithuanian patients with different clinical outcomes**

Trait or marker	Clinical diagnosis		
	Duodenal ulcer	Chronic gastritis	P
*cag*PAI$^+$	75.7% (28/37)	45.5% (20/44)	<0.01
*cag*PAI$^-$	24.3% (9/37)	54.5% (24/44)	<0.01
vacA s1	75.7% (28/37)	40.9% (18/44)	<0.01
vacA s2	24.3% (9/37)	59.1% (26/44)	<0.01
vacA s1/m1	16.2% (6/37)	11.4% (5/44)	>0.05
vacA s1/m2	32.5% (12/37)	18.2% (8/44)	>0.05
vacA s2/m2	24.3% (9/37)	56.8% (25/44)	<0.05
vacA s1/s2-m?	27.0% (10/37)	13.6% (6/44)	>0.05
*cag*PAI$^+$/*vacA s1*	75.7% (28/37)	38.6% (17/44)	<0.001
*cag*PAI$^+$/*vacA s2*	0%(0/37)	6.8% (3/44)	NS
*cag*PAI$^-$/*vacA s1*	0% (0/37)	2.3% (1/44)	NS
*cag*PAI$^-$/*vacA s2*	24.3% (9/37)	52.3% (23/44)	<0.05
iceA1	46.0% (17/37)	40.9% (18/44)	NS
iceA2	43.2% (16/37)	54.6% (24/44)	NS
iceA?	10.8% (4/37)	4.5% (2/44)	NS

NS – not significant.

response (23, 24). We ascertained that around two-thirds of Lithuanian *H. pylori* strains (59%) have *cag*PAI, and our data support previous reports from Western countries and suggest that persons colonized with *cag*PAI-positive *H. pylori* strains are at increased risk of developing peptic ulceration (24, 25).

The *vacA* gene is present in essentially all strains of *H. pylori*, but its nucleotide sequence varies among strains. Our study confirmed a strong association between the *vacA* genotype and cytotoxin phenotype of *H. pylori* strains. Our results are in agreement with other reports (6, 26) indicating a higher prevalence of the *vacA s1* allele in patients with DU. Some previous studies (26, 27) suggested that the *vacA m1* subtype of the *vacA* gene might be a suitable marker for the increased virulence of *H. pylori*. Our study has revealed that s2/*m2* strains are more characteristic for chronic gastritis; however, we have not found that the *H. pylori vac*A *s1/m1* subtype is associated with more severe outcome of *H. pylori* infection. The PCR-based typing system of the *vacA* middle-region failed to classify all Lithuanian strains. This unsuccessful PCR typing is probably due to mutations within primer regions and indicates diversity in the *vacA* middle-region sequence in different communities (28, 29).

Geographic differences have also been important in distribution of the *vacA* genotypes. The majority of *H. pylori* isolates from patients of Lithuania had the *s1a* subtype. This finding is in agreement with the earlier reports suggesting *s1a* predominance in Northern and Eastern Europe (30, 31). However, only two Lithuanian *H. pylori* strains showed *vacA s1b* genotype, and *vacA s1c* subtype was not detected, which is common in South Africa and East Asia, respectively, but infrequent in Europe (32, 33).

With regard to *iceA* gene, it was reported that *iceA1* allele was related to the peptic ulcer disease in the United States (34), and the Netherlands (5); however, this finding has not been confirmed in other countries, such as Japan (16, 33), India (35), and Korea (16). Our study has not revealed an association between the *iceA* genotype and more severe disease outcome in Lithuanian patients. As reported earlier, *H. pylori* should not be considered as a single infectious organism, but as a worldwide population of bacterial variants, which might have different clinical impact in different parts of the globe (8).

Conclusion

Results of our study suggest that *cag*PAI and *vacA s1* genotypes are associated with peptic ulceration in Lithuanian patients infected by *Helicobacter pylori*.

Helicobacter pylori lietuviškųjų padermių genotipai sergantiesiems lėtiniu gastritu ir dvylikapirštės žarnos opalige

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Raktažodžiai: *Helicobacter pylori*, dvylikapirštės žarnos opaligė, lėtinis gastritas, virulentiškumo veiksniai, *cag*PAI, *vacA*, *iceA*.

Santrauka. *Darbo tikslas. Helicobacter pylori* (*H. pylori*) infekcijos sukeltų ligų pobūdis gali būti susijęs su šios bakterijos padermių virulentiškumu. Įvairiuose geografiniuose regionuose *H. pylori* padermių paplitimas skiriasi. Šio tyrimo tikslas – nustatyti *H. pylori* padermių genetines charakteristikas (*cag*PAI, *vacA*, *iceA* genus) dvylikapirštės žarnos opalige ir lėtiniu gastritu sergantiems ligoniams iš Lietuvos.

Tirtųjų kontingentas ir tyrimo metodai. Ištirti 37 dvylikapirštės žarnos opalige ir 44 lėtiniu gastritu (funkcine dispepsija) sirgę ligoniai. *H. pylori* padermėms, išaugintoms iš endoskopinio tyrimo metu paimtos skrandžio gleivinės biopsinės medžiagos, *cag*PAI, *vacA* (*s* ir *m* potipių) ir *iceA* genai buvo tiriami polimerazių grandinės reakcijos metodu, naudojant specifinius pradmenis.

Rezultatai. Tyrimų rezultatai rodo, kad 59,3 proc. *H. pylori* padermių turėjo su citotoksinu susijusį A geną (*cag*PAI). Iš opalige sergančių pacientų 75,7 proc. buvo infekuoti *cag*PAI turinčiomis *H. pylori* padermėmis, o tarp sergančiųjų lėtiniu gastritu tokių ligonių buvo tik 45,5 proc. ($p<0,01$). Visoms tirtoms 81 *H. pylori* padermėms radome *vacA* geną. Tarp sergančiųjų opalige 75,7 proc. buvo infekuoti *vacA s1* genotipo padermėmis, o sergantieji lėtiniu gastritu – 40,9 proc. ($p<0,01$). Tarp sergančiųjų lėtiniu gastritu skirtingai nei opalige dažnesnės buvo *vacA s2/m2* genotipo *H. pylori* padermės (56,8 proc. ir 24,3 proc., $p<0,05$). Padermių, turinčių *iceA1* genotipą, dažnis tiriamųjų grupėse nesiskyrė (46,0 proc. ir 40,9 proc., $p>0,05$).

Išvada. H. pylori infekuotiems ligoniams šios bakterijos *cag*PAI ir *vacA s1* genotipų padermės yra glaudžiai susijusios su dvylikapirštės žarnos opalige.

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Received 14 April 2008, accepted 12 June 2008
Straipsnis gautas 2008 04 14, priimtas 2008 06 12