Comparative analysis of selected cagPAI genes and different vacA genotypes in Iranian and Turkish H. pylori-positive patients suffering from gastric adenocarcinoma and active chronic gastritis

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Background/aim: Helicobacter pylori is a pathogen that colonizes a majority of the world's population. Genetic diversity within the virulence genes of bacteria such as cagPAI and vacA may have a modified effect on the pathogenic potential of the bacteria. This study aimed to investigate which genes can be suggested as potentially related virulence factors for H. pylori-associated active chronic gastritis and stomach adenocarcinoma in the northwest of Iran and south of Turkey.

Materials and methods: Formalin-fixed, paraffin-embedded stomach biopsy tissue samples were obtained from Iranian and Turkish patients from selected geographical regions. The prevalence of selected cagPAI genes and vacA genotypes were studied in H. pylori-positive samples by using polymerase chain reaction and specific primers.

Results: Out of 320 patients, H. pylori was detected in 28.43% of patients. We found that the vacAs1, vacAm2, and cagA genes with mean prevalences of 82.41%, 71.42%, and 69.23%, respectively, were dominant in Iranian and Turkish patients.

Conclusion: In the south of Turkey and northwest of Iran the studied genes were homogeneous and there were no significant differences in bacterial genetics. The results of this study indicate that cagA and vacAs1 are dominant genes in people with gastric disorders in our selected geographical regions.

Key words: cagPAI, Helicobacter pylori, vacA, adenocarcinoma, chronic gastritis

1. Introduction

Today's understanding of Helicobacter-related gastric diseases in humans stems from an explosion in research that occurred after the first culture of the organism by Marshall and Warren in 1982 (1). Several risk factors for gastric cancer have been identified but the clinical outcome of Helicobacter pylori infection depends on host and bacterial factors. Helicobacter is a genus of gram-negative Epsilonproteobacteria usually found in the stomach. It is a human pathogen responsible for chronic active gastritis; infection by this organism is an important risk factor for peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma (2,3,4). As symptoms are often absent or nonspecific in patients in the early stages of the disease, gastric cancer is usually diagnosed at an advanced stage, when curative options are limited. With exceptions in countries that have developed screening programs for early diagnoses, most patients with cancers reach treatment already in advanced stages (5). The prevalence of H. pylori infection varies between countries; generally, the prevalence is about 30% in developed and more than 80% in developing countries (6). However, the incidence of the cancer varies from region to region.

In recent years, many H. pylori virulence factors have been characterized. The cytotoxin-associated gene pathogenicity island (cagPAI) and vacuolating cytotoxin gene A (vacA) are two identified virulence factors that are considered to have an important role in the pathogenesis of H. pylori infection (7). cagPAI is a nearly 40-kb cluster of genes and is the most studied marker of H. pylori. There are
31 open reading frames predicted within the cag region. One of these open reading frames encodes the immune dominant antigen CagA, which is localized to the -3’ end of the island. CagA was identified as the first protein of cagPAI and appeared to be a major virulence factor (8). On the other hand, allelic variation can be seen in the vacA genotypes. Allelic variation among *Helicobacter pylori* vacA occurs in both the signal sequences (s region) and the midregion (m region) of the gene. Strains with genotype slm1 produce high levels of vaculating cytoxin in vitro (9,10). However, strains with genotype s2 produce an inactive toxin (11).

2. Materials and methods

2.1. Sample collection

Our study was performed on 320 formalin-fixed, paraffin-embedded (FFPE) tissues obtained from patients from the northwest of Iran and south of Turkey. For the Turkish population 80 paraffin blocks from patients with gastric adenocarcinoma and 80 paraffin blocks from patients with a nonadenocarcinoma diagnosis (chronic gastritis) were obtained from the pathology archive of the Pathology Department of the Medical Faculty of Çukurova University (Balcalı Hospital) according to the histopathological diagnosis reports. For the Iranian population the same numbers of samples as mentioned above were taken from the pathology archive of the Pathology Department of the Medical Faculty of Tabriz University of Medical Sciences (Imam Reza Hospital).

In order to determine the minimum amount of samples to obtain sufficient DNA for analysis, we collected five to seven 5-µm-thick cut sections from each selected paraffin block by using a new disposable blade for each sample on a semiautomatic microtome and placed them into 2.0-mL polypropylene microcentrifuge tubes for DNA extraction. Deparaffinization (paraffin removal) procedures as a preextraction treatment were used for FFPE tissues according to guidelines by using xylene. To remove the residual xylene, the samples were washed several times with descending concentrations of ethanol. Finally, the tubes were kept open for evaporation of remaining ethanol (12). The DNA extraction procedure was performed with the QIAamp DNA Kit (QIAGEN Inc., Germany) according to the manufacturer’s instructions. Prepared DNAs were stored at −20 °C. The total amount and purity of DNA for each sample were assessed by spectrophotometry (CHE BIOS, UV/Vis spectrophotometer). The total amount of DNA was obtained in ng/µL, and the A260/280 ratio was calculated for protein impurities. DNA was considered viable for amplification when the A260/A280 ratio value was from 1.7 to 2.0 (13).

2.2. PCR

PCR analyses were carried out to determine the presence or absence of cagA, cagE, cagT, cagG, cagM, vacA s1, vacA s2, vacA m1, and vacA m2 genes in each *H. pylori*-positive sample. All PCR mixtures were performed in a total volume of 25 µL. The glmM gene (urea C) was primarily amplified for detection of *H. pylori* DNA in our samples (14,15). In the rest of the study the amplification reactions for each positive sample for the glmM gene were performed by using specific primers and protocols (thermocycler: MJ Mini, Bio-Rad) for the detection of the considered genes (15–18). The sequences of primers used in this study and sizes of amplicons are described in Table 1.

*H. pylori* 26695 DNA was used as a positive control for cagPAI-positive strains (14). *H. pylori*-positive strains for genes selected in this study were used as a control for all the reactions performed (16). All stages of this study were approved by the Ethics Committee of the Faculty of Medicine, Çukurova University, Adana, Turkey.

2.3. Detection of PCR products

For analysis of the amplified products of each PCR assay, 6 µL of the amplicons were electrophoresed with a 1X Tris-acetate-EDTA buffer on 2% agarose gel stained by ethidium bromide (5 µL/100 mL). The amplicons were visualized by UV transillumination and a 100-bp ladder was used as standard.

3. Results

From the 320 patients, *H. pylori* (presence of glmM) was detected in 91 patients (28.43%). Detailed demographic data for the presence of different cagPAI genes and vacA genotypes and histopathological findings are shown in Table 2 and Figures 1 and 2. Subsequently, combinations of genotypes were made and then compared between the two groups of patients, those with gastric adenocarcinoma and nonadenocarcinoma diagnosis, in our study to understand the existence of probable relationships between the presence of various genes and the clinical and histopathological outcomes of diseases. Associations between the presence of cagPAI selected genes, vacA subtypes, and outcomes of disease are shown in Table 2. Statistical differences in demographic characteristics among the different disease groups were determined by chi-square test (Table 2).

4. Discussion

Since the discovery of *H. pylori*, several studies have focused on elucidating the microorganism’s pathogenicity mechanisms associated with clinical outcome. Gastric cancer is one of the most common worldwide cancers and is a highly lethal disease. Establishment of *H. pylori* as one of the risk factors for this kind of malignancy helps to identify high-risk individuals; however, infection with this organism is very common and most colonized people never develop cancer. Thus, techniques to identify high-
**Table 1.** Description of the pairs of primers used in the amplification of cagPAI and vacA genes.

<table>
<thead>
<tr>
<th>Gene/primers</th>
<th>Sequence</th>
<th>Size/references</th>
</tr>
</thead>
</table>
| glmM         | F: AAG CTT TTA GGG GTG TTA GGG GTT T  
               R: AAG CTT ACT TTC TAA CAC TAA CGC | 294 bp (16,29,30) |
| cag A        | F: GAT AAC AGGCAA GCT TTT GAG G  
               R: CTG CAA AAG ATT GTT TGG CAG A | 349 bp (16,17,25) |
| cag E        | F: TTGAAAACTTCAAGGATAGGATAGGAC  
               R: GCGCTAGCTAATATCACCAATTACCC | 508 bp (15,27,29) |
| cag T        | F: CCATGTTTATACGGGCTGTGT  
               R: CATCACCAACACCTTT TGAT | 301 bp (16) |
| cag G        | F: GCCATGTTAACACCCCCCTAG  
               R: ATTTTCAACAAAGTTAGAAAGCC | 497 bp (17) |
| cag M        | F: ACAAAATCAAAAAAGAAAAAGAGGCC  
               R: ATTTTTCAACAAAGTTAGAAAGCC | 587 bp (17) |
| vacA s1      | F: GTC AGC ATC ACA CCG CAA C  
               R: CTG CTT GAA TGC GCC AAA C | 190 bp (30–32) |
| vacA s2      | F: GCT AAC ACG CCA AAT GAT CC  
               R: CTG CTT GAA TGC GCC AAA C | 199 bp (31) |
| vacA m1      | F: GGTTAATATACGCGGTATG  
               R: CATTGTTAATAGTAAAC | 290 bp (13,31) |
| vacA m2      | F: GGA GCC CCA GGA AAC ATT G  
               R: CAT AAC TAG CGC CTT GCA C | 352 bp (13,32) |

**Table 2.** Association between the presence of cagPAI selected genes, vacA subtypes, and disease outcome in Iranian and Turkish patients.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>A.Ch.G</th>
<th>Cancer</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Iran (n = 27)</td>
<td>Turkey (n = 28)</td>
<td>X²</td>
</tr>
<tr>
<td>cagA</td>
<td>22 (81.48%)</td>
<td>16 (57.14%)</td>
<td>3.813</td>
</tr>
<tr>
<td>cagE</td>
<td>10 (37.0%)</td>
<td>9 (32.1%)</td>
<td>0.146</td>
</tr>
<tr>
<td>cag T</td>
<td>7 (25.92%)</td>
<td>8 (28.57%)</td>
<td>0.049</td>
</tr>
<tr>
<td>cag G</td>
<td>3 (11.11%)</td>
<td>3 (10.71%)</td>
<td>0.002</td>
</tr>
<tr>
<td>cag M</td>
<td>1 (3.70%)</td>
<td>1 (3.57%)</td>
<td>0.001</td>
</tr>
<tr>
<td>vacA s1</td>
<td>22 (81.48%)</td>
<td>22 (78.57%)</td>
<td>0.073</td>
</tr>
<tr>
<td>vacA s2</td>
<td>6 (22.22%)</td>
<td>6 (21.42%)</td>
<td>0.005</td>
</tr>
<tr>
<td>vacA m1</td>
<td>7 (25.92%)</td>
<td>7 (25%)</td>
<td>0.006</td>
</tr>
<tr>
<td>vacA m2</td>
<td>21 (77.7%)</td>
<td>22 (78.57%)</td>
<td>0.005</td>
</tr>
<tr>
<td>vacA s1/m1</td>
<td>6 (22.22%)</td>
<td>7 (25%)</td>
<td>0.059</td>
</tr>
<tr>
<td>vacA s2/m1</td>
<td>14 (51.85%)</td>
<td>15 (53.57%)</td>
<td>0.016</td>
</tr>
<tr>
<td>VacA s1/m1</td>
<td>1 (3.70%)</td>
<td>1 (3.57%)</td>
<td>0.001</td>
</tr>
<tr>
<td>vacA s2/m1</td>
<td>6 (22.22%)</td>
<td>7 (25%)</td>
<td>0.059</td>
</tr>
</tbody>
</table>
risk subpopulations must utilize other biological markers. The prevalence of vacA genotypes and cagPAI component genes in H. pylori isolates from different parts of the world are various, and there is a direct association between specific genotypes and certain clinical appearance. cagA, as a marker for the presence of cagPAI, is one of the best studied virulence factors for H. pylori. The frequency of cagA-positive isolates has been reported to be nearly 25%–60% in Bahrain, Israel, and Jordan and 60%–80% in some other countries such as Taiwan, Turkey, Malaysia, and India (19). According to one article, the cagA genotype varied geographically from 44% to 94% in Iranian populations (19). In the present study, we used simple PCR for structural screening of selected cagPAI genes and vacA genotypes in clinical isolates of H. pylori in FFPE gastric biopsy specimens obtained from 320 patients suffering
from active chronic gastritis and gastric adenocarcinoma. The samples were obtained from two reference hospitals in the northwest of Iran (Imam Reza Hospital) and south of Turkey (Balcali Hospital) (160 samples from reference hospitals of each country, comprising 80 chronic gastritis and 80 gastric adenocarcinoma samples), as two neighboring developing countries where the prevalence of *H. pylori* infection can be as high as 85%. *H. pylori* was detected in 33.75% and 35% of Iranian and Turkish patients with chronic gastritis and in 21.25% and 23.75% of Iranian and Turkish patients with gastric adenocarcinoma, respectively. The mean frequency of *H. pylori* infection was 28.44% in all samples.

Based on previously published data, the prevalences of *cagA* in Iranian isolates were 62%, 92%, and 68.7% in Tehran, Jahrom, and Tabriz, respectively (20–23). According to statistical analysis, in our study area in Iran, there was no significant association between the presence of selected *cagPAI* genes, *vacA* subtypes, and gastric adenocarcinoma. However, the prevalence of some selected genes was questionably high in patients suffering from stomach adenocarcinoma and active chronic gastritis. In a recent study by Ghotaslou et al. in 2013, 68.7% of the Iranian patients were infected with *cagA*-positive strains. On the other hand, in a current study from Turkey, Karaman et al. reported a *cagA* positivity rate of 65.5% and they also found a significant relationship between *cagA* status and peptic ulcer disease (22). Saltık et al. reported the *cagA* positivity rate as 55.6% in 45 isolates and they found no significant difference between *cagA* positivity and the severity of gastroduodenal symptoms (24). It is known that *cagA* positivity rates and their association with clinical outcomes differ from region to region. For example, studies from East and South Asian countries show that more than 90% of the strains carry the *cagA* gene regardless of clinical outcome. In general, the *cagA* prevalence rate has been found to be between around 50% to 70% in Middle Eastern countries, whereas in East Asian countries almost all isolated strains are *cagA*-positive. According to our data the *cagA* positivity rate in Turkey for chronic gastritis and gastric adenocarcinoma was 57.14% and 78.94%, respectively, and these rates in Iranian patients were 81.48% and 58.8%.

The chaperone-like protein CagT plays an essential role in the translocation of *cagA* into host cells (25) and our results showed that the prevalence of *cagT* in Iranian strains was lower than in Turkey. This may be one of the reasons why *cagA* does not play a clear role in the formation of gastric adenocarcinoma in Iran.

Like *cagA*, *cagE* belongs to the *cagPAI* and it is responsible for binding to cell receptors and inducing the release of IL-8 (26). In our samples *cagE* was found in 52.94% and 57.89% of Iranian and Turkish adenocarcinoma samples respectively and for chronic gastritis samples it was 37% and 32.1% in Iranian and Turkish patient samples, which reflects an important result, showing that this gene, like *cagA*, or in cooperation with *cagA*, may be related to the formation of active chronic gastritis and gastric cancer development in both countries. This study demonstrates that it is probable that infection with a *cagE*-positive *H. pylori* strain is associated with gastric disorders such as gastritis and adenocarcinoma in Iranian and Turkish patients in the selected areas. We found that *cagE*, not only *cagA*, could be used as a marker for the presence of *cagPAI* in our selected geographical regions.

Although the prevalence of strains positive for *cagE*, *cagT*, *cagM*, *vacAs1*, and *vacAm2* in Iran and Turkey is similar, we can suggest that *vacAs1*, *vacAm2*, and *cagA* positivity be considered as essential virulence factors for the development of most severe gastric diseases, like gastric adenocarcinoma, in the Iranian and Turkish populations.

The results of the present study demonstrate that *vacA* subtypes s1 and m2 are dominant in Iran and Turkey, similar to other Middle East countries, and the frequencies of s1m2 in patients with active chronic gastritis and s1m1 in patients with adenocarcinoma were relatively high. The s2m2 genotypes were much lower compared to other publications from Iran (27%) and also around the world (0%–57%) (27).

Due to limitations in obtaining fresh endoscopy samples from patients, we used paraffin-embedded tissue blocks. Although formalin fixation with paraffin embedding is the universal method for tissue preservation and stabilization prior to histological evaluation by pathologists (28), the effectiveness of PCR using DNA gained from FFPE tissue is affected by multiple factors, including the type of fixative used, the fixation time, the DNA extraction method, the length of the PCR target, the concentration of DNA, and the PCR protocol itself. Low percentages of extracted DNA and false negative responses are thus the main disadvantages of this method.

In conclusion, similar to studies performed in the Middle East, the association between *cagA* and *cagE* positivity and the virulence of *H. pylori* strains was remarkable among patients from the northwest of Iranian and south of Turkey with different disease outcomes. According to information obtained from this investigation, we can conclude that in patient groups selected from the south of Turkey and northwest of Iran it seems that the genes that were studied are homogeneous and in this era there is no significant difference in bacterial genetics. The results of this study indicate that *cagA* and *vacAs1* are dominant in our studied area. However, in this population there is no statistically significant association between these factors and gastric cancer. It is possible that if the
statistical population increases, the association of cagA with cancer might be meaningful. It can be concluded that with the exception of H. pylori infection other factors such as host genetics and nourishment play important roles in the formation of gastric cancer in the populations of our studied areas from Iran and Turkey. Considering the gap of information observed during our research relating to genotyping and other aspects of H. pylori infection, in order to achieve our goals further advanced molecular investigations like DNA sequencing analysis and studies of larger statistical populations from different geographical regions from Iran and Turkey are recommended.

References


