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Determination of correlation between principal genotypes of *Helicobacter pylori* according to cagPAI components and *vacA* genotypes and clinical out come in patients suffering from active chronic gastritis and gastric adenocarcinoma from Iran and Turkey.

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Abstract

Objective(s): *Helicobacter pylori* are gastric infectious agents that colonizes majority of the world's population. Genetic diversity among the virulence factors of bacterium like cytotoxin associated gene Pathogenicity Island (cagPAI) and vacuolating cytotoxinA (vacA) could have a modifying result on the pathogenic potential of the infecting strain. This study aimed to analyse which genes can be recommended as doubtless related virulence factors for *H. pylori* associated active chronic gastritis and stomach adenocarcinoma in Iranian and Turkish population.

Material and methods: We tend to targeted on some cag PAI components and *vacA* gene subtypes based on correlations shown in some previous studies. So as to realize our goal, formalin fixed Paraffin Embedded (FFPE) tissues obtained from Iranian and Turkish patients. The prevalence of the cagPAI and vacA genotypes were studied in *H. pylori* positive samples by using Polymerase Chain Reaction (PCR) technique and specific primers.

Results: From all of 320 patients, *H. pylori* were detected in 28.43% of patients. We tend to found that *vacAs1*, *vacAm2* and *cagA* genes with mean prevalence of 82.41%, 71.42% and 69.23% were dominant in both of Iranian and Turkish patients.

Conclusion: Finally in Turkish and Iranian population the genes that were studied, was homogeneous and there's no important variations in bacterial genetic and with the exception of *H. pylori* infection different factors like host genetic and nourishment play a crucial role within the formation of gastric cancer. However it's attainable that if statistical population will increase, the *cagA* gene association with cancer are going to be meaningful.

Keywords: Cytotoxin associated gene Pathogenicity Island (cagPAI), *H. pylori*, Vacuolating cytotoxinA (vacA), Adenocarcinoma, Chronic gastritis.

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Introduction

Today's understanding of *Helicobacter*-related gastric diseases in humans stems from an explosion in research, which occurred after the first culture of the organism by Marshall et al. [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* found usually in the stomach. It is a human pathogen responsible for chronic active gastritis; infection with this organism is an important risk factor for peptic ulcer, gastric cancer and gastric mucosa-associated lymphoid tissue lymphoma [2-4]. As symptoms are often absent or nonspecific in patients with the early stages of the disease, gastric cancer is usually diagnosed in an advanced stage, when curative options are limited. With exceptions in

countries that have developed screening programs for early diagnoses, most patients reach treatment with cancers 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. Cytotoxin associated gene Pathogenicity Island (cag-PAI) and vacuolating cytotoxin geneA (*vacA*) are two identified virulence factors that are considered to have an important role in the pathogenesis of *H. pylori* infection [7]. The cytotoxin-associated gene island also referred to as the cag-PAI is a nearly 40 kb cluster of genes and is the most studied marker of the *H. pylori*. There are 31 open reading frames predicted within the cag region. One of these open reading frames encodes the immunodominant antigen CagA, which is localized to the -3' end of the island. CagA was identified as the first protein of the cag PAI and appeared to be a major virulence factor [8]. On the other hand, the 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 the genotype s1m1 produce high levels of vacuolating cytotoxin *in vitro* [9,10]. However, strains with the genotype s2 produce an inactive toxin [11].

Materials and Methods

Sample collection

Our study performed on 320 Formalin Fixed Paraffin Embedded (FFPE) tissues obtained from patients from Iran and Turkey. For Turkish population, 80 paraffin blocks from patients with gastric adenocarcinoma and 80 paraffin blocks from patients with non-adenocarcinoma diagnosis (chronic gastritis) were obtained from pathology archive of the pathology department of Medical Faculty of Cukurova University according to their histopathological diagnosis report. Furthermore, for Iranian population another group of samples as same as samples mentioned above were taken from pathology archive of the Pathology Department of Medical Faculty of Tabriz University of Medical Sciences. 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 with using a new disposable blade for each sample on a semi-automatic microtome and placed them into a 2.0 ml polypropylene microcentrifuge tubes for *H. pylori*-DNA extraction. Deparaffinization (paraffin removal) procedures as a pre-extraction 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 the remaining ethanol evaporation [12]. The DNA extraction procedure was performed with QIAamp DNA Kit (Qiagen Inc. Germany) according to the manufacturer's instructions. Prepared DNA was stored at -20°C.

Evaluation of extracted DNA

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 A260/280 ratio value was from 1.7 to 2.0 [13].

PCR assays

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* (urease 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 glmM gene were performed by using specific primers and protocols (Thermo cycler: MJ Mini Bio-Rad) for detection of considered genes [15-18]. The sequences of primers used in this study and size of amplicons are described in Table 1.

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

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 base pair ladder was used as standard.

Results

From all of 320 patients, *H. pylori* (presence of glmM) were detected in 91 patients (28.43%). Detailed demographic data of presence of different cag PAI and vacA subtypes and histopathological findings are shown in Table 2. Subsequently, the combinations of genotypes were made and then compared the two groups of patients, with gastric adenocarcinoma and non-adenocarcinoma diagnosis in Iranian and Turkish patients of our study in order to understand the existence of probable relationship between the presence of various genes and the clinical and histopathological outcome of diseases. Association between the presences of cag PAI selected genes, *vacA* subtypes and disease outcome shown in Table 2. Statistical differences in demographic characteristics among the different disease groups were determined by chi-square test.

Table 1. *Description of the pairs of primers used in the amplification of cag PAI and vacA genes.*

Gene/ Primers	Sequence	Size/References
glmM	F: AAG CTT TTA GGG GTG TTA GGG GTTT R: AAG CTT ACT TTC TAA CAC TAA CGC	294 bp [16,20,28]
cag A	F: GAT AAC AGG CAA GCT TTT GAG G R: CTG CAA AAG ATT GTT TGG CAG A	349 bp [16,17,24]
cag E	F: TTGAAAACTTCAAGGATAGGATAGAGC R: GCCTAGCGTAATATCACCATTACCC	508 bp [15,20,29]
cag T	F: CCATGTTTATACGCCTGTGT R: CATCACCACACCCTT TTGAT	301 bp [16]
cag G	F: GCCATGTTAACACCCCCTAG R: TTAATGCGCTAGAATAGTGC	497 bp [17]
cag M	F: ACAAATACAAAAAAGAAAAAGAGGC R: ATTTTTCAACAAGTTAGAAAAAGCC	587 bp [17]
vacA s1	F: GTC AGC ATC ACA CCG CAA C R: CTG CTT GAA TGC GCC AAA C	190 bp [25,30,31]
vacA s2	F: GCT AAC ACG CCA AAT GAT CC R: CTG CTT GAA TGC GCC AAA C	199 bp [25]
vacA m1	F: GGTCAAAATGCGGTCATGG R: CCATTGGTACCTGTAGAAAC	290 bp [13,25]
vacA m2	F: GGA GCC CCA GGA AAC ATT G R: CAT AAC TAG CGC CTT GCA C	352 bp [13,27]

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

Genotypes	A. Ch. G				Cancer				Total % (n=91)
	Iran% (n=27)	Turkey% (n=28)	$\chi 2$	P-value	Iran% (n=17)	Turkey% (n=19)	χ^2	P-value	
CagA	22 (81.48)	16 (57.14)	3.813	0.05	10 (58.8)	15 (78.94)	1.712	0.1907	63 (69.23)
CagE	10 (37.0)	9 (32.1)	0.146	0.702	9 (52.94)	11 (57.89)	0.089	0.7652	39 (42.85)
CagT	7 (25.92)	8 (28.57)	0.049	0.8257	4 (23.52)	5 (26.31)	0.037	0.8472	24 (26.37)
CagG	3 (11.11)	3 (10.71)	0.002	0.9624	2 (11.76)	1 (5.26)	0.496	0.4811	9 (9.89)
CagM	1 (3.70)	1 (3.57)	0.001	0.9791	1 (5.88)	0	1.15	0.2836	3 (3.29)
VacAs_1	22 (81.48)	22 (78.57)	0.073	0.7874	14 (82.35)	17 (89.47)	0.047	0.8277	75 (82.41)
VacAs_2	6 (22.22)	6 (21.42)	0.005	0.9432	0	0	-	-	12 (13.18)
VacAm_1	7 (25.92)	7 (25)	0.006	0.9372	6 (35.29)	5 (26.31)	0.341	0.5593	25 (27.47)
VacAm_2	21 (77.77)	22 (78.57)	0.005	0.9432	11 (64.70)	11 (57.89)	0.048	0.8259	65 (71.42)
VacAs_1/m_1	6 (22.22)	7 (25)	0.059	0.8085	6 (35.29)	7 (36.84)	0.009	0.9231	26 (28.57)
VacAs_1/m_2	14 (51.85)	15 (53.57)	0.016	0.8984	11 (64.70)	12 (63.15)	0.056	0.8134	53 (58.24)
VacAs_2/m_1	1 (3.70)	1 (3.57)	0.001	0.9791	0	0	-	-	2 (2.19)
VacAs_2/m_2	6 (22.22)	7 (25)	0.059	0.8085	0	0	-	-	13 (14.28)

Discussion

Gastric cancer is one of the most common cancers worldwide 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 of colonized persons never develop cancer. Thus, techniques to identify high-risk subpopulations must utilize other biological markers. Since the discovery of *H. pylori*, several studies have focused on demonstration of the microorganism pathogenicity mechanisms association with distribution of particular virulence genes (mainly vacA alleles and the presence of *cagPAI* genes) in clinical outcome of *H. pylori* infection in different geographical regions. Nevertheless, the clinical relevance of these putative virulence-associated genes of *H. pylori* is still a matter of controversy.

The prevalence of vacA genotypes and cagPAI component 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. This study was designed to characterize the genotype of *H. pylori* with using simple PCR method for structural screening of selected *cag-PAI* genes and vacA genotypes in FFPE gastric biopsy specimens obtained from 320 patients which suffering from chronic gastritis and Gastric adenocarcinoma. These samples obtained from Iran and Turkey (160 samples from each country, covering 80 chronic gastritis and 80 gastric adenocarcinoma samples), as two neighbour developing countries where the prevalence of the *H. pylori* infection can be as high as 85%. *H. pylori* was detected in 33.75% (27/80) and 35% (28/80) of Iranian and Turkish patients with chronic gastritis, and in 21.25% (17/80) and 23.75% (19/80) of Iranian and Turkish patients with

gastric adenocarcinoma respectively. The mean frequency of *H. pylori* infection was 28.44% in all samples.

It has frequently been described that the effectiveness of PCR with using gained DNA 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. So low percentages of extracted DNA in this method can described with DNA damages during fixation and extraction steps [19]. But in fresh samples DNA extraction rate is high. Lima et al. reported *H. pylori* infection in 94 out of 101 (93.1%) patients with gastric carcinomas and they found *cagE* gene in 53.2% of *H. pylori*-positive gastric cancer cases [20].

The cagA, as a marker for the presence of the cag PAI, is one of the best studied virulence factors for *H. pylori* and 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 [22]. According to an article, the cagA positive genotype varied geographically from 44% to 94% in Iranian populations [22]. Also based on previously published data, the prevalence of cagA in Iranian isolates were 62%, 92% and 68.7% in Tehran, Jahrom and Tabriz, respectively [23-25]. In a recent study by Ghotaslou et al., 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 [25]. But Saltik et al. reported the cagA positivity rate as 55.6% in 45 isolates and they found no significant difference between the *cagA* positivity and the severity of the gastro duodenal symptoms [27]. It has been known that cagA positivity rates and their association with clinical outcomes differ from region to region. For example studies from East to South Asian countries shows that more than 90% of the strains carry the *cagA* gene regardless of clinical outcomes. In general, the cagA prevalence rate has been found to be between around 50% and 70% in Middle Eastern countries, whereas in the East Asian countries almost all isolated strains are cagA positive [28]. According to our data cagA positivity rate in Turkey for chronic gastritis and gastric adenocarcinoma was 57.14% and 78.94% respectively and this rate in Iranian patients was 81.48% and 58.8%.

Like cagA, cagE belongs to cagPAI and is responsible for binding to cell receptors and inducing the release of interleukin-8. 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 with formation of active chronic gastritis and gastric cancer development in both countries. This study demonstrates that infection with a cagE positive *H. pylori* strain was associated with gastric disorders such as gastritis and adenocarcinoma in Iranian and Turkish patients. We found that cagE, but not only cagA, could be used as a marker for the presence of cag PAI in our geographical region. The other virulence factor of *H. pylori* that most studied is chaperone-like protein; CagT plays an essential role in the translocation of cagA into host cells [29]. In the present study our results showed that the prevalence of cagT in Turkish and Iranian patients with chronic gastritis was 28.57% and 25.92 respectively and it was 26.31% and 23.52% for gastric adenocarcinoma samples.

According to our data cagG positive *H. pylori* strains were isolated from 11.11% and 10.71% of Iranian and Turkish patients with chronic gastritis and in 11.76% and 5.26% of Iranian and Turkish patients with gastric adenocarcinoma respectively. In our study the prevalence of cagM in Iranian and Turkish patients was not significant but CagE and CagM are absolutely necessary for IL-8 secretion. The different combinations of vacA s and m regions identify the virulence characteristic of the *H. pylori* strains. It has been reported that type s1m1 strains produce a higher cytotoxic activity *in vitro* than type s1m2 strains, while s2m2 strains, which are considered as fewer virulent strains, produce no detectable cytotoxin. Thus, identification of vacA profiles of the isolated strains and evaluation of these subtype combinations together with the clinical outcome in patients have significant importance. In accordance with the previous reports from Turkey and Iran, we found vacA s1 as the predominant vacA s subtype with a rate of 81.48% and 78.57% in Iranian and Turkish patients with chronic gastritis respectively [25]. This rate for patients with gastric adenocarcinoma was 82.35% and 89.47%. According to the reports, in all isolated *H. pylori* strains from East Asian countries, vacA s1 and cagA were positive, where the gastric cancer incidence has been reported in higher rates [25]. So our finding supports the idea that the strains with vacA s1 genotype secrete the toxin more effectively.

According to our data, m1 positive genotypes were detected in 25.92% and 25% of Iranian and Turkish patients with chronic gastritis and in 35.29% and 26.31% of Iranian and Turkish patients with gastric adenocarcinoma respectively. Also m2 genotypes detected in 77.77% and 78.57% of Iranian and Turkish patients with chronic gastritis and it was 64.70% and 57.89% for Iranian and Turkish patients with gastric cancer. We found the s1m1 strains prevalence in 22.22% and 25% of Iranian and Turkish patients suffering from chronic gastritis and this rate for Iranian and Turkish patients with gastric adenocarcinoma were 35.29% and 36.84% respectively. Also s1m2 strains were predominantly detected in isolates from chronic gastritis. Our findings were similar with the previous reports done by Nagiyev et al. from Turkey [29,30]. These findings also are consistent with the findings of Ghotaslou et al. from Iran [24]. The prevalence of vacA s2/m1 genotype was very low in selected samples from both countries (3.70% and 3.57%), and we did not find s2/m1 and s2/m2 strains in Iranian and Turkish patient samples with gastric adenocarcinoma.

Although the prevalence of cagE, cagT, cagM, vacAs1 and vacAm2 positive strains in Iran and Turkey is approximately similar but we can suggest that, vacAs1, vacAm2 and cagA

positivity, can be considered as an essential virulence factor for the development of most severe gastric diseases, like gastric adenocarcinoma in Iranian and Turkish population. The results of present study demonstrates that, vacA subtypes s1 and m2 are dominant in Iran and Turkey, similar to other Middle East countries and the frequency of s1m2 strains in patients with active chronic gastritis and s1m1 strains in patients with adenocarcinoma were relatively high in both countries. The s2m2 genotypes were much lower unlike the other publications from Iran (27%) and also around the world (0%-57%) [26]. However, the current study does not rule out an association between the expression of vacA subtypes or *cagPAI* genes and the virulence of *H. Pylori* but according to statistical analysis, in our study in Iran and Turkey, there was no significant association between the presence of selected *cagPAI* genes, vacA subtypes and gastric adenocarcinoma and chronic gastritis. But prevalence of some selected genes was questionable high in patients suffering from stomach Adenocarcinoma and active chronic gastritis.

Conclusion

According to information obtained from this investigation, we can conclude that, as in other populations, cagPAI and vacA genotype variations can be used as predictive markers in *H. pylori* clinical isolates to identify a particular strain as a gastritis or cancer producer. Similar to studies performed in Middle East, the association between cagA and cagE positivity and virulence of *H. pylori* strains was remarkable among Iranian and Turkish patients with different disease outcomes. Also we can conclude that in Turkish and Iranian population the genes that were studied, was homogeneous and in this area there is no significant difference in bacterial genetics and in studied population there is no statistically significant association between selected factors and gastric cancer. But it is possible that if statistical population increases, association of cagA with gastric cancer will be meaningful (p=0.050). In a nutshell, it can be concluded that with the exception of *H. pylori* infection other factors such as host genetics and nourishment play an important role in the formation of gastric cancer in Iranian and Turkish population. 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 studying on larger statistical populations recommended.

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