RESEARCH ARTICLE

Investigation of Association between oipA and iceA1/iceA2 Genotypes of *Helicobacter pylori* and Gastric Cancer in Iran

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Abstract

Background: H pylori is the main causative agent of Gastric cancer and chronic gastritis. Genetic diversity of H. pylori has major contribution in its pathogenesis. We investigated the prevalence of oipA and iceA1/iceA2 positive strains of *H. pylori* among patients with gastric cancer and gastritis. Materials and Methods: Sampling performed by means of endoscopy from 86 patients. DNA was extracted from tissue samples using DNA extraction kit. PCR assay was performed and products were monitored by Agarose Gel Electrophoresis. Results: Urease Test and 16S rRNA PCR did not show significant differences in detection of *H. pylori*. The frequency of iceA1 allele in patients with gastric cancer was significantly higher than those with gastritis ($p<0.05$). However, there was no significant difference in prevalence of oipA and iceA2 genes among the two groups of patients ($p>0.05$). Conclusions: The iceA1 gene, but the oipA and iceA2 genes , is associated with *H. pylori*-induced gastric cancer. However, confirmatory studies must be performed in future.

Keywords: Chronic gastritis - gastric cancer - genetic diversity - *Helicobacter pylori*

Asian Pac J Cancer Prev, **15 (19)**, 8295-8299

Introduction

Gastric cancer, with poor prognosis, occurs in any part of the stomach, and after lung cancer, is second cause of caner death worldwide (Correa, 2013). Several factors thought to be associated with gastric cancer development; including *Helicobacter pylori* (*H pylori*) infection, genetic background, sex, age, diet, smoking, and etc. (de Martel et al., 2013). *H pylori*, previously called Campylobacter pylori, is the main causative agent of Gastric cancer and chronic gastritis (Konturek, 2003). This bacterium was first found in patients with chronic gastritis and gastric ulcers (Marshall, 2001). It is reported that more than 50 percent of people have *H pylori* infection, among which, however, only some develop gastroentric diseases (Rothenbacher and Brenner, 2003; Kusters et al., 2006). In addition to the immune system status and genetic predisposition of the host, many virulence factors of *H pylori* influence the development and progression of *H pylori*-related diseases (Wroblewski and Peek, 2013).

Several investigations have suggested that genetic diversity and substantial heterogeneity of *H pylori* has major contribution in pathogenesis of this bacterium, as each genotype leads to different type of diseases (Go et al., 1996; Marshall et al., 1996). For instance, cagA gene of *H pylori* is revealed to be involved in pathogenesis of Gastric cancer (Graham and Yamaoka, 1998; Zhang et al., 2013). In addition, cagA and the vacuolating cytotoxin (vacA) genotypes are proposed to serve as predictors for progression of gastric lesions (Gonzalez et al., 2011). Moreover, presence of other genes of *H pylori* such as iceA (induced by contact with epithelium), oipA (outer inflammatory protein), and babA (blood group antigen–binding adhesin), has been reported to be associated with development of gastroentric disorders (Kim et al., 2001; Markovska et al., 2011). Most of these genes encode the proteins that help *H pylori* interact with host or change host cellular homeostasis, which ultimately lead to abnormalities like neoplastic tissues (Ilver et al., 1998; Naumann, 2005).

OipA, also known as HopH, is a member of outer membrane proteins (OMPs) family 1 that is encoded by HP0638/hopH gene of *H pylori* (Alm et al., 2000). OipA is shown to be associated with increased secretion of interleukin 8, progression of gastric inflammation, bacterial adherence and adaptation to the host microenvironment (Yamaoka et al., 2000; 2002; Dossumbekova et al., 2006), and thereby, contribute to development of gastroentric diseases. For instance, Markovska and colleagues (2011) observed that 97% of Bulgarian patients with peptic ulcers

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and 66% of those with Gastritis were oipA positive. In addition, Dabiri and colleagues (2009) reported that oipA was more prevalent in Iranian patients with Gastric cancer than in those with peptic ulcers.

In 1998, Peek and colleagues discovered of a novel *H pylori* gene, iceA, by comparing mRNA transcripts from an ulcer-derived and a gastritis- derived strain of *H pylori*, and revealed two distinct alleles of iceA1 and iceA2, following DNA sequences (Peek et al., 1998). The iceA expression is induced by the contact with epithelium during the attachment of *H pylori* to the gastric mucosa; as reported by van Doorn and colleagues (1998) both iceA1 and iceA2 alleles were expressed in gastric biopsies specimens. Furthermore, it is observed that iceA1 expression is associated with increased mucosal concentrations of IL-8 and enhanced mucosal inflammation (Peek et al., 1999). Results of investigation by Ciftci and colleagues (2011) in patients with gastric cancer and chronic gastritis suggested geographical differences in contribution of iceA1 and iceA2 alleles to clinical outcomes

In present study, we attempted to investigate and compare the prevalence of oipA and iceA1/iceA2 positive strains of *H pylori* among patients Gastric cancer and gastritis from two hospitals of Tabriz city located in East Azarbaijan Province of Iran. We aimed to find out whether there is an association between above-mentioned genes of *H pylori* and Gastric cancer or not.

Materials and Methods

Study patients

Participants consisted of 86 patients, 41 women and 45 men, referring to the Gastroenterology and Hepatology Centers of Imam Reza and Shahid Madani Hospitals of Tabriz, capital of East Azarbaijan Province of Iran; between September 2012 and May 2013. Demographic information of the participants was collected using a questionnaire. The patients' age ranged from 19 to 87 years; 37 of them suffered from Gastric cancer (mean age 68.62±12.36) and 49 from acute or chronic Gastritis (mean age 41.55±14.69). Individuals with atrophic Gastritis and also those were who had received medications such as; anti-Helicobacter, anti-inflammatory, and non-steroidal drugs during three months prior to endoscopy were excluded from the study. The ethical committee of the Hospitals approved the use of the clinical information and the collection of samples for research purposes. All participants signed a written informed consent letter.

Endoscopy and biopsy sampling

Two gastric antral biopsy specimens were taken from each patient by means of Endoscopy; one was then used for rapid-Urease test, and the other was stored in -80°C for PCR assay. For every patient, distinct sampling forceps was used, and after every endoscopy the endoscope's tube was washed and sterilized using an automatic washing system.

DNA extraction

DNA was extracted from biopsy specimens using DNA extraction DNGTM-Plus kit (Cinna Gen Co., Iran), according to the manufacturer's instruction. In details, the DNA extraction solution was incubated in Bain Marie at 37°C for 20 min. Each specimen was transferred on a sterile Lam, grinded smoothly using a scalpel, and returned to its pertinent microtube. Afterwards, 500 μl of DNGTM-Plus solution was added on a ground specimen, and the microtube was vortexed until the tissue was dissolved to obtain a completely homogenous suspension. Next, 300 μl of Isopropanol (at -20°C) was added and vortexed for 5 seconds, and incubated at -20°C for 20 min. After that, the tubes were centrifuged at 12000 rpm for 10 min, the supernatant was discarded lightly, and the pellet-containing microtubes were kept inverted on a filter paper for 2-3 seconds. The pellet was then re-suspended in 1ml of 75% ethanol, vortexed and centrifuged at 12000 rpm for 5 min. This step was repeated once more, and then the pellet was incubated at 65°C, until the alcohol was removed. After dryness, 50 μl of distilled water was added, followed by incubation at 65°C for 5 min and centrifuge at 12000 rpm for 30 seconds. Finally, the supernatant containing extracted DNA was transferred to a 0.5-ml-microtube, and was stored at 4°C for one day, and then at -20°C until PCR performance.

Agarose Gel Electrophoresis for 45 minutes was used for qualification of extracted DNA, using TAE buffer, ethidium bromide 1% and gel documentation (GELDOC) system (UVltedc Co., UK). In addition, purity of the DNA was measured by spectrophotometry at 260 and 280 nm, optimized for DNA and protein, respectively, and A 260/280 ratio was calculated. For determining the concentration of DNA, 1/500 and 1/1000 dilutions of DNA in distilled water were used for spectrophotometry only at 260 nm, and the concentration was calculated by following formula: Concentration of ds DNA (ng/μl) =OD *Dilution *50

Primers and PCR Assay

As given in Table 1, primers specific for16S rRNA (Westbrook et al., 2005), and iceA1, iceA2 and oipA genes (Ben Mansour et al., 2010) were recruited for genotyping of *H pylori* using PCR method. PCR assay was performed by a 25-well thermo-cycler (Eppendorf) in a reaction volume of 25μL, using a commercially available kit (CinnaGen, Iran). The PCR conditions for all studied genes included; 95°C for 5min, thirty-five cycles at 94°C for 50 s, 56°C for 50 s, and 72°C for 50 s, and finally followed by one cycle at 72°C for 5 min.

PCR products were then monitored for presence/absence and quantity of desired genes by performing 1.2% Agarose Gel Electrophoresis (Mod. SH-505). Briefly, PCR products were mixed with loading buffer with ratio of 6/1, and run on the gel using voltage 100-110 for 20-25 min. After that, the product was stained with ethidium bromide solution for 10 min, and then was visualized by UV transilluminator.

Statistical analysis

The data were analyzed using SPSS v19 software. The relationship between the frequencies of each allele with the risk of gastric cancer, and the differences in frequency

Table 1. Characteristics of the Oligonucleotide Primers Used for Genotyping of *H. pylori*

Gene	Primer	Name	Length	GC%	Tm °C	Sequence
16S rDNA	Forward	HP1	22 nt	45.45%	58.33	5'-GCAATCAGCGTCAGTAATGTTC-3'
	Reverse	HP2	22 nt	50.00%	57.81	5'-GCTAAGAGATCAGCCTATGTCC-3'
oipA	Forward	HPO638F	20 nt	35%	52.92	5'-GTTTTTGATGCATGGGATTT-3'
	Reverse	HPO638R	20 nt	45%	54.75	5'-GTGCATCTCTTATGGCTTTG-3'
iceA1	Forward	iceA1F	20 nt	30%	48.15	5'-GTGTTTTTAACCAAAGTATC-3'
	Reverse	iceA1R	20 nt	45%	54.57	5'-CTATAGCCAGTCTCTTTGCA-3'
iceA2	Forward	iceA2F	21 nt	28.57%	48.88	5'-GTTGGGTATATCACAATTTAT-3'
	Reverse	iceA2R	21 nt	38.10%	53.64	5'-TTGCCCTATTTTCTAGTAGGT-3'

Table 2. Comparison of Sensitivity of Urease Test and 16S rRNA PCR for Detection of *H pylori* Infection in Patients with Gastric Cancer and Gastritis

Detection Method	Gastric Cancer (n=37)			Gastritis (n=49)		
	Women	Men	Total	Women	Men	Total
Urease Test	9 (24.3%)	19 (51.4%)	28 (75.7%)	17 (34.7%)	15 (30.6%)	32 (65.3%)
16S rRNA PCR	9 (24.3%)	21 (56.8%)	30 (81.1%)	18 (36.7%)	17 (34.7%)	35 (71.4%)
p value	1	0.641	0.572	0.833	0.667	0.515

Table 3. Differences between Frequencies of *H pylori* Infection in Patients with Gastric Cancer and Gastritis

Disease	Urease Test			16S rRNA PCR		
	Women	Men	Total	Women	Men	Total
Gastric Cancer (n=37)	9 (24.3%)	19 (51.4%)	28 (75.7%)	9 (24.3%)	21 (56.8%)	30 (81.1%)
Gastritis (n=49)	17 (34.7%)	15 (30.6%)	32 (65.3%)	18 (36.7%)	17 (34.7%)	35 (71.4%)
P value	0.3	0.05	0.3	0.22	0.041	0.302

Table 5. Presence of oipA, iceA1 and iceA2 genes of *H pylori* in Women and Men with Gastric Cancer and Gastritis

Disease	Gender	*H pylori* (+)	oipA (+)	iceA1 (+)	iceA2 (+)
Gastric Cancer	Women (n=15)	9 (60.0%)	7 (46.7%)	5 (33.3%)	2 (13.3%)
	Men (n=22)	21 (95.5%)	10 (45.5%)	8 (36.4%)	10 (45.5%)
	P value	0.007	0.942	0.85	0.04
gastritis	Women (n=26)	18 (69.2%)	12 (46.2%)	5 (19.2%)	7 (26.9%)
	Men (n=23)	17 (73.9%)	8 (34.8%)	2 (8.7%)	5 (21.7%)
	P value	0.717	0.419	0.293	0.674

Table 4. Frequency of oipA, iceA1 and iceA2 Genes of *H pylori* in Patients with Gastric Cancer and Gastritis

Disease	*H pylori* (+)	oipA (+)	iceA1 (+)	iceA2 (+)
Gastric Cancer (n=37)	30(81.1%)	17(45.9%)	13(35.1%)	12(32.4%)
Gastritis (n=49)	35(71.4%)	20(40.8%)	7(14.3%)	12(24.5%)
p value	0.302	0.634	0.023	0.416

of genes between patients and controls (patients with gastritis) were evaluated by chi-square, and proportional tests. The differences with $p<0.05$ were considered as statistically significant.

Results

Presence of H pylori in gastric specimens

Results of Urease test and 16S rRNA PCR for detection of *H pylori* did not show significant differences ($p>0.05$). As revealed by Urease test, 75.7% of patients with Gastric cancer and 65.3% with gastritis were infected with *H pylori*, while 16S rRNA PCR detected this species in 81.1% and 71.4% of patients with Gastric cancer and gastritis, respectively (Table 2). As depicted in Table 3, there were no significant differences between frequencies of *H pylori* infection in patients with Gastric cancer and gastritis ($p>0.05$). However, a significant difference was seen in frequency of *H pylori* infection in men with Gastric cancer and gastritis, detected by 16S rRNA PCR ($p<0.05$).

Prevalence of H pylori oipA genotype

By using specific primers for oipA gene (HPO638F and HPO638R), presence of this gene was verified in 45.9% of *H pylori*-positive patients with Gastric cancer and in 40.8% of *H pylori*-positive patients with gastritis (Table 4); there was no significant difference in prevalence of oipA gene between two groups of the patients ($p>0.05$). These findings indicate that there is no association between oipA genotype of *H pylori* and Gastric cancer. In addition, no significant difference was observed prevalence of oipA genotype between women and men ($p>0.05$) among both patients with Gastric cancer and gastritis (Table 5).

Frequency of iceA1 and iceA2 Alleles

Results of PCR assay for iceA1 and iceA2 alleles disclosed that 35.1% of *H pylori* isolates from patients with Gastric cancer and 14.3% with gastritis had iceA1 genotype, and that 32.4% of patients with Gastric cancer and 24.5% with gastritis had iceA2 genotype. As shown in Table 4, the prevalence of iceA1 allele in the patients with Gastric cancer was significantly higher than those with gastritis ($p<0.05$), which indicates that there may be

an association between iceA1 allele and gastric cancer. However, no association was found between iceA2 allele and Gastric cancer. Furthermore, no significant difference was seen in frequency of iceA1 allele between women and men in the case of either Gastric cancer or gastritis (p>0.05). However, frequency of iceA2 allele in men with Gastric cancer (but not gastritis) was significantly higher than women with Gastric cancer (p<0.05).

Discussion

We have recently demonstrated that the vacA d1 genotype of *H pylori* may help predict risk for gastric adenocarcinoma and peptic ulcer disease in Tabriz, East Azerbaijan of Iran (Basiri et al., 2014). In this study we investigated the prevalence of *H pylori*, oipA gene, iceA1 and iceA2 alleles in biopsy specimens of patients with Gastric cancer and gastritis from Tabriz.

Urease Test is a common approach to detect *H pylori* in clinical samples. However, PCR method is more sensitive, but expensive tool for determination of this bacterium presence in clinics (Lage et al., 1995). We used Urease test for primary detection of *H pylori*, and to approve its results, we recruited 16S rRNA PCR. Our findings indicated no significant differences in sensitivity of Urease test and PCR for detection of *H pylori* which may be explained by our small sample size.

Several studies among different geographical populations have shown that there might be association between infection with *H pylori* and Gastric cancer development (Thomazini et al., 2006; Zhang et al., 2013). Thomazini and colleagues (2006), for instances, observed that 95% of Brazilian patients with Gastric cancer had infection with H. pylori. Zhang and colleagues (2013) reported that among 184 Korean patients with Gastric cancer, 89.1% were *H pylori* positive. On the other hand, many studies reported that the prevalence of *H pylori* among patients with Gastric cancer is lower compared to those with chronic gastritis and Gastric ulcers (Martins et al., 2005; Chomvarin et al., 2008). We found *H pylori* infection in higher percentage of patients with gastritis compared to patients with Gastric cancer; however this difference was not significant. In addition, we found that higher prevalence of *H pylori* among men may develop Gastric cancer, in comparison with women that seemed to develop gastritis, although these variations were not significant and this hypothesis need to go under further investigations.

The oipA gene encodes an extracellular inflammatory protein that is considered as a virulence factor for *H pylori* (Kudo et al., 2007). It is found that oipA gene is present in samples from stomach of significant percentage of patients with gastritis and gastric ulcers (Salih et al., 2007). Moreover, some investigations have reported this gene in and proposed its association with Gastric cancers. According to Ben Mansour and colleagues (2010), using PCR, 95.3% of patients with Gastric cancer, and 80.7% of patients with gastric ulcers revealed infection with *H pylori* oipA genotype, which indicated an association between presence of this gene and development of gastric cancer. In contrast, Yamaoka and colleagues (2002) observed oipA in 67% of patients with Gastric cancer in Unite states of America, and reported no association between oipA and development of Gastric cancer. In the present study, although we detected oipA in 56.6% of patients with Gastric cancer, we found no association between presence of oipA gene of *H pylori* and development of Gastric cancer. Our finding was in agreement with that of Yamaoka and colleagues (2002), and in disagreement with finding of Ben Mansour and colleagues (2010). We also observed that gender factor might have no effect on infection with *H pylori* oipA genotype and on its role in Gastric cancer development.

The iceA1 allele has been reported to be associated with enhanced mucosal inflammation and development of Gastric diseases (Peek et al., 1999; Vega et al., 2010). Ciftci and colleagues (2011) reported that iceA1 genotype of *H pylori*, compared to iceA2 genotype, was more prevalent among patients with chronic gastritis and gastric cancer patients. On the contrary, Liu and colleagues (2012) demonstrated that, as a pathogenic mechanism of gastric diseases, *H pylori* virulence genes were more relevant than colonization density and that among isolates from patients with Gastric cancer, 26% were of iceA1 genotype and 46% of iceA2 genotype. Our findings showed that both iceA1 and iceA2 alleles were predominant in patients with Gastric cancer, and that there might be an association between iceA1 allele and gastric cancer. However, no association was observed between iceA2 allele and gastric cancer. These findings are in agreement with Ciftci and colleagues (2011), and in disagreement with Liu and colleagues (2012). Furthermore, our results indicated that frequency of iceA2 allele in men with Gastric Cancer was significantly higher than women which may be an insight into etiology of Gastric diseases associated with *H pylori* infection.

In conclusion, results of our investigation indicated that prevalence of *H pylori* does not differ among patients with Gastric cancer and gastritis. Importantly, we found that there might be an association between iceA1 genotype of *H pylori* and development of Gastric cancer. However, we could not find any association between oipA gene and iceA2 genotypes of *H pylori* and pathogenesis of Gastric cancer. To our knowledge, there are a few evidences for role of iceA1 allele and other genotypes of *H pylori* in pathogenesis of Gastric cancer. Thus, our findings need to be further verified through large-scaled studies, in which different virulent factors and their interactions should be simultaneously studied.

Acknowledgements

We kindly thank all patients and participants of this study. We are also thankful from Dr. Farzam Ajamian, Dr. Hamid Reza Vaziri, and Dr. Mohammad Hossein Somi for their consultation and help during the sampling. This study was financially supported by University of Tabriz, and was performed in Imam Reza hospital of Liver and Gastrointestinal Disease Research Center at Tabriz University of Medical Sciences, Tabriz, Iran.

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