RESEARCH ARTICLE

Genotyping of Peroxisome Proliferator-Activated Receptor gamma in Iranian Patients with *Helicobacter pylori* Infection

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Abstract

***Helicobacter pylori* (*H. pylori*) infection as a serious problem in both adults and children can induce chronic gastritis, peptic ulcer disease (PUD), and possibly gastric cancer. The aim of the current study was to survey antibiotic resistance and also to determine influence of PPARγ polymorphism in patients with *H. pylori* infection. During an 11-month-period, 98 *H. pylori* isolates were collected from 104 biopsy specimens. *In vitro* susceptibility of *H. pylori* isolates to 4 antimicrobial agents metronidazole, clarithromycin, amoxicillin and tetracycline were assessed by quantitative method according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline. PPARγ polymorphism was determined using polymerase chain reaction-restriction fragment length polymorphism assay. The frequency of *H. pylori* infection in our study was 94.2%. *In vitro* susceptibility data showed that highest level of resistance was related to metronidazole (66.3%), and the majority of *H. pylori* isolates were highly susceptible to amoxicillin and tetracycline (94.9% and 96.9%, respectively). Genotypic frequencies were 25.5% for CC (Pro12Pro), 40.8% for GC (Pro12Ala) and 33.7% for GG (Ala12Ala). In our study, CG genotype had highest distributions among infected patients with *H. pylori*. The study suggests that the PPAR-γ Pro12Ala polymorphism could be evaluated as a potential genetic marker for susceptibility to gastric cancer in the presence of *H. pylori* infection.**

Keywords: Peroxisome proliferator-activated receptor γ - *Helicobacter pylori* - gastric cancer

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Introduction

Helicobacter pylori (*H. pylori*) is a gram-negative, rod-shaped, flagellate, microaerophilic spiral bacillus and one of common bacterial infections in the world that colonizes the stomachs of about 50-60% of the world's population (Parsonnet et al., 1991). Infection with the bacteria is important public health problems in developing countries as well as in developed countries (Frenck Jr and Clemens, 2003). Person-to-person contacts, oral-oral and fecal-oral routes are major routes of transmission. Effective antibacterial therapy in order to eradication of infection is necessary. Combination therapy consisting of a proton pump inhibitor with a macrolide and a β-lactam as an eradication regiment could be effective for treatment of *H. pylori* infections, but antimicrobial resistance has been reported by several investigators (Eun et al., 2003). *H. pylori* is responsible for a spectrum of infections that can be ranged from mild or chronic gastritis to peptic ulcer, gastric lymphoma, and gastric cancer (Vilaichone et al., 2014).

Gastric cancer is the second most common cancer in the world and long-standing infection with *H. pylori* is linked to gastric cancer. However, exact mechanism that underlying to *H. pylori*-associated gastric carcinogenesis is still poorly understood (Uemura et al., 2001, Eun et al., 2003). It is clear that the pathogenicity of *H. pylori* is not only explained by bacterial virulence factors alone. Recently, several researchers have proved the role of host genetic factors involved in the *H. pylori*-associated gastric carcinogenesis (Tahara et al., 2008; Shibata et al., 2010; Goto et al., 2011; Jing et al., 2012).

The peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily, are ligand-dependent transcription factor that play an important role in cellular differentiation and carcinogenesis as well as regulation of fatty acid oxidation and glucose utilization. To date, three different PPAR subtypes (α, β, and γ) have been described (Michalik et al., 2004). Human PPARγ gene, located on chromosome 3, is consists of 9 exons (A1, A2, B, and 1-6), from which the two isoforms PPARγ1 and PPARγ2 are generated through alternative

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promoter usage by differential splicing of at least in three different transcripts from the PPAR γ gene on chromosome 3p25 (Greene et al., 1994; Michalik et al., 2004; He, 2009).

Human PPARγ expression was first described in hematopoietic cells and then in spleen, liver, testis, skeletal muscle and brain He (2009). PPARγ plays many functional roles in different organs and tissues (Michalik et al., 2004). Several studies demonstrated that PPARγ expression increases during *H. pylori* infection. Several polymorphisms in PPARγ2 have been identified, but of which the most common is a CCA to GCA missense mutation in codon 12 of exon B of the PPARγ gene. These polymorphisms can change protein structure and reduce function of the PPARγ 2 genes (Greene et al., 1994). Several *in vitro* studies have found that the PPARγ, Pro12Ala polymorphism confers protection against diabetes and colorectal cancer (Greene et al., 1994; Michalik et al., 2004). Although it is found that PPARγ Pro12Ala polymorphism is associated with gastric cancer risk, but it is not completely understood. The aim of present study was to determine antimicrobial susceptibility and PPARγ polymorphism in patients with *H. pylori* infection.

Materials and Methods

Study population and sampling

In this cross sectional study, a total of 104 biopsy specimens were collected from patients with suspected *H. pylori* infection who were referred to gastroenterology wards of Tehran (capital city of Iran) hospitals during December 2012 to October 2013. Patients who had history of previous use of nonsteroidal anti-inflammatory drugs (NSAIDs) and proton pomp inhibitors in three weeks before proceeding to endoscopy were excluded from the study. Two biopsy specimens were taken from each patient. One of them was used for rapid urease test and the other was immediately transported to the laboratory in Thioglycolate Broth (Merck, Germany) supplemented with 3% yeast extract. *H. pylori* was identified by inoculation of grind biopsies on Brucella blood agar (Merck, Germany) containing defibrinated sheep blood (5%), vancomycin (5 mg/L), trimethoprim (5 mg/L), and polymyxin B (0.25 mg/L). The plates were incubated in microaerophilic conditions (5% O_2, 10% CO_2, 85% N2) at 37°C for 3-7 days. *H. pylori* was identified by gram stain, culture, oxidase, catalase and rapid urease test.

Antibiotic susceptibility test

Determination of antimicrobial susceptibility was performed by estimating minimum inhibitory concentration (MIC) method according Clinical Laboratory and Standards Institute (CLSI) criteria. Four antimicrobial drugs (Metronidazole, Clarithromycin, Amoxicillin and Tetracycline) that were purchased from Sigma-Aldrich (St. Louis, Mo) were used in this study. The MIC was defined as the lowest concentration of each antimicrobial agent that inhibited visible growth of the tested isolate (10). The ranges of MIC value used in this study for metronidazole were 0.25 to 32 μg/ml; clarithromycin 0.125-4μg/ml, tetracycline 0.125-16 μg/ml and amoxicillin 0.125-16 μg/ml. MIC breakpoint for all antibiotics was recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). MIC50 and MIC90 were estimated for each antibacterial drug and it is define as the minimum concentration that necessary to inhibit the visible growth of 50% and 90% of microorganisms.

DNA extraction and PCR assay

Template DNA was extracted using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's procedure. The concentration of extracted DNA was measured spectrophotometrically. PCR was carried out for detection of PPARγ gene. The sequences of PCR primers were: forward 5´-GCCAATTCAAGCCCAGTC-3´ and reverse 5´-GATATGTTTGCAGACAGTGTATCAGTGAAGG AATCGCTTTCCG-3´. PCR conditions for amplification of 270 bp fragments of the PPARγ was done by thermocycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation for 3 min at 94ºC, 35 cycles of denaturing at 94ºC for 45 seconds, annealing at 55ºC for 45 seconds, and extension at 72ºC for 90 seconds. The final extension was carried out at 72ºC for 10 minutes.

PPARγ polymorphism

After amplification, to determine PPARγ polymorphism, digestion was performed with 10μl of PCR product, 3 μl of enzyme buffer and 5 units of Bst UI restriction enzyme (Fermentas, Vilnius, Lithuania) for 8 hours at 60ºC according to the manufacturer's recommendation. The DNA fragments were separated on 1% agarose gel (Invitrogen, Carlsbad, CA, USA) prepared in TAE buffer at 80V for 2 hours and visualized using ultraviolet light (UVItec, Cambridge, UK) after staining with ethidium bromide. The size and number of generated fragments after digestion with restriction endonuclease enzyme were as follow: undigested fragment at 270 bp (Pro12Pro, CC genotype), two digested fragments at 227 and 43 bp (Ala12Ala , GG genotype) and three digested fragment at 270, 227 and 43 bp (Pro12Ala, GC genotype). For confirmation of the RFLP results, each experiment was repeated twice.

Results

During the 11-months study period, a total of 98 patients with *H. pylori* infection were included in this study (median age 49±5.9 years, 60 males (61.2%) and 38 female (38.8%)). Out of 98 patients 35 (35.7%) had gastric cancer, 22 (22.5%) duodenal ulcer, 19 (19.4%) peptic ulcer, 17 (17.3%) gastric ulcer and gastric cancer with peptic ulcer (5.1%). The frequency of *H. pylori* infection in our study population was 98 out of 104 samples (94.2%). Nineteen isolates (19.4%) were susceptible to all the tested antibiotics. Increased resistance to tetracycline and amoxicillin was observed for 3 (3.1%) and 5 (5.1%) of isolates respectively. 55 (84.6%) of all the isolates resistant to metronidazole, had MIC ≥ 16 μg/ml and the rest of isolates (15.4%) had MIC ≥ 32 μg/ml. three (3.1%) of the isolates were resistant to metronidazole (MIC ≥32

Table 1. Antimicrobial Susceptibilities of 98 Helicobacter Pylori isolates to 4 Antimicrobial Agents

agent	MIC(μg/ml)			No.(%)of isolates		MIC Interpretive Breakpointsa (R/S)
	Range	50%	90%	S	R	
Metronidazole	0. 25-32	16	16	33 (33.7)	65 (66.3)	≤8>
clarithromycin	0.125-4	0.5	0.5	80 (81.6)	18 (18.4)	≤0.25/0.5≥
tetracycline	0.125-16	1	1	95 (96.9)	3 (3.1)	≤1≥
amoxicillin	0.125-16	0.5	0.5	93 (94.9)	5 (5.1)	≤0.12≥

[a]MIC breakpoint for all antibiotics was recommended by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org)

Table 2. Distribution of Different Genotype of PPARγ in Patients with Helicobacter Pylori

PPARγ genotype	gastric cancer (n=35)	duodenal ulcer (n=22)	peptic ulcer (n=19)	gastric ulcer (n=17)	gastric cancer + peptic ulcer (n=5)
CC	-	2 (9.1%)	11(57.9%)	12(70.6%)	-
CG	22(62.8%)	2(9.1%)	5(26.3%)	1(5.9%)	3
GG	13(37.2%)	18(81.8%)	3(15.8%)	4(23.5%)	2

μg/ml), were isolated from patients with gastric cancer and peptic ulcer. All of *H. pylori* strains were inhibited by tetracycline at MIC ≤0.5 μg/ml (except three of them). Resistance to metronidazole and clarithromycin was observed in 9 isolates (9.2%). PPARγ polymorphism was investigated in all 98 patients. PPARγ genotype frequencies among patients were as follow: CC genotype (25.5%), GG genotype (33.7%) and GC genotype (40.8%). In compare with other PPARγ genotypes, CG genotypes had highest distributions among infected patients with *H. pylori*. The higher frequency of GC genotype was seen in gastric cancer patients and higher frequency of GG genotype was also seen in duodenal ulcer patients. None of gastric cancer patients had PPARγ CC genotype. The relationship between PPARγ Pro12Ala GC genotypes and gastric cancer were statistically significant (P<0.02).

Discussion

H. pylori is considered as the major cause of disorders such as duodenal ulcer, peptic ulcer, gastric cancer in both children and adults than can lead to gastric cancer. It is reported that proper regimen can lead to 85-90% eradication rate (Marshall et al., 1987). Therefore, awareness about antibiotic resistance profile of *H. pylori* to prescript the most effective therapy regimen is necessary. According to the literatures, the rate of metronidazole resistance varies from 10% to 80% and is gradually increasing in different geographic regions (Kim et al., 2001). In our study, highest rate of resistance was related to metronidazole (66.3%). In 2010, Haghi et al. reported that the rates of resistance to metronidazole, clarithromycin, amoxicillin and tetracycline in 128 *H. pylori* isolated from biopsy specimens were 64%, 23%, 2.5% and 0%, respectively (Tomatari et al., 2010). In other study that was done in Korea, 1118 *H. pylori* isolated from gastric biopsies were investigated between 1997 and 2001. They exhibited that resistance to metronidazole, has increased from 25.2% in 1997 to 71.4% in 2000 (Ling et al., 2002). Chisholm et al. in a study done in England and Wales over a 6-year period (2000-2005) showed that the rate of resistance was varied from 28.6% to 36.3% for metronidazole and 8.3% to 12.7% for clarithromycin (Chisholm et al., 2007). Another study that was done on 2204 clinical isolates of *H. pylori* in 18 European countries, during a 3-years period, the rate of resistance to metronidazole, clarithromycin and levofloxacin was 34.9%, 17.5% and 14.1% respectively (Megraud et al., 2013). In our study, the resistance rate to metronidazole was lower than Columbia (82%) (Alvarez et al., 2009), Mexico (76.3%) (Torres et al., 2001) and Sweden (76%) (Wheeldon et al., 2004). Increase of resistance to metronidazole among our isolates could be mediated by high prescription of this antibiotic in treatment of parasitic, gynecological and dental disease (Gerrits et al., 2006) and intrinsic pathways (decreased drug uptake or increased drug efflux) and genetic pathways (rdxA, frxA and fdxB genes) (van Amsterdam et al., 2005).

According to the several studies, resistance to clarithromycin in different geographic area is gradually increasing (Ling et al., 2002). In our study, the rate of resistance to clarithromycin was 18.4%, which was lower than Turkish (27.6%) (Baglan et al., 2006) and in one European study (43.5%) (van Doorn et al., 2001), but was higher than Korea (8.3%) (Ling et al., 2002), and England (12.7%) (Chisholm et al., 2007). This variation in resistance to clarithromycin might be related to widespread use of erythromycin in treatment of common infections, cross resistance with other macrolides and point mutations (Mansour et al., 2010). The results of this study showed remarkable activity of tetracycline and amoxicillin against all of tested isolate (MIC90, 1 and 0.5 μg/ml, respectively). In accordance with recent data, resistance to amoxicillin and tetracycline was rare (Tomatari et al., 2010). Although in compared with metronidazole and clarithromycin, resistance to amoxicillin and tetracycline is low, it should be assessed periodically. Therefore, it is important that the antimicrobial susceptibility testing to select a valuable treatment plans for *H. pylori* infections to be performed.

As mentioned, PPARγ as a host gene plays important role in *H. pylori*-associated gastric carcinogenesis and

also has strong effects on various diseases including regulation of adipocyte differentiation, fatty acid uptake and storage, atherosclerosis, inflammation, osteoporosis, type-2 diabetes mellitus, insulin resistance and obesity. According to the previous studies, polymorphism in PPARγ especially G genotype or Ala allele in codon 12 may increase risk of distal gastric cancer (LIAO et al., 2006; Canbay et al., 2012). In our study, frequency of GG genotype and GC genotype were 33.7% and 40.8%, respectively.

Results from a case-control study in China (2006), to determine PPARγ polymorphism, indicated that frequency of PPARγ allele G (Ala12) was significantly higher among cancer patients than in the control group. They found that combination of PPARγ G allele and *H. pylori* infection further increased the risk of gastric cancer and the presence of the Ala12 did not increase the risk of gastric cancer in *H. pylori*-negative subjects (LIAO et al., 2006). In other case-control study by Canbay et al. conducted in Turkey (2012), 68 patients with gastric cancer and 129 controls were assessed. They exhibited that PPARγ Pro12Ala gene polymorphism was associated with gastric cancer and carriage of G genotype (Ala 12) allele had increased risk (1.95 fold) for gastric cancer (Canbay et al., 2012). In a study conducted in India between 2002 and 2007, Prasad et al. reported 58.6% prevalence of *H. pylori* infection from 348 patients enrolled in their study (62 gastric adenocarcinoma, 45 peptic ulcer and 241 non ulcer dyspepsia). This study confirmed that Pro12Ala PPARγ polymorphism in the presence of *H. pylori* infection could be a marker for genetic susceptibility to gastric adenocarcinoma and PUD (Prasad et al., 2008). In accordance with the results of recent studies in Germany (Konturek et al., 2004), Japan (Tahara et al., 2008), Turkey (Canbay et al., 2012), China (LIAO et al., 2006), India (Prasad et al., 2008) and Iran (Bazargani et al., 2010), our results revealed that PPARγ Pro12Ala polymorphism may be a potential genetic marker for susceptibility to gastric cancer in the presence of *H. pylori* infection.

This study showed relatively high resistance to metronidazole among our isolates. Tetracycline and amoxicillin had remarkable activity against *H. pylori* infection. The present study demonstrated that Pro12Ala PPARc polymorphism may be considered as a risk factor of gastric cancer in presence of *H. pylori* infection. Therefore, Pro12Ala PPARγ polymorphism might be as a predicting factor for gastric cancer in presence of *H. pylori* infection.

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