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Full Length Research Paper

Polymerase chain reaction-restriction fragment length polymorphism analysis of *Helicobacter pylori* isolates from patients with gastrointestinal complaints in Eastern Turkey

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The aim of this study was to investigate the genetic variations among isolates of *Helicobacter pylori* (*H. pylori*) obtained from patients with gastritis (G) and peptic ulcer (PU) in Elazig Province, East of Turkey and to determine association between restriction fragment length polymorphism (RFLP) types and clinical/histopathological outcomes. Determining the dominant genotypes among *H. pylori* isolates would detect potential risk and aid in the development of improved treatment and control strategies. Sixty one *H. pylori*-isolates [29 males and 32 females, 51 with G, 10 with PU (duodenal ulcer (DU) or gastric ulcer (GU)] were examined by polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) for detecting the genetic diversity among *H. pylori* isolates. By (PCR-RFLP) analysis, *H. pylori* isolates yielded five (A-E) different band profiles. The most common type of *H. pylori* isolates from patients with chronic active, chronic active non-atrophic and chronic atrophic G was type B (55, 88.9 and 100%, respectively). However, most of the isolates from patients with DU and GU were type D (75 and 100%, respectively). To the best of our knowledge, this is the first report on PCR-RFLP analysis of *H. pylori* isolates in Elazig Province, the East of Turkey. This study indicated that the genetic heterogenecity among *H. pylori* isolated from patients is low, and the clinical/histopathological outcomes of *H. pylori* isolates associated to the RFLP types.

Key words: *Helicobacter pylori*, patients, genetic variation, polymerase chain reaction-restriction fragment length polymorphism.

INTRODUCTION

Helicobacter pylori (*H. pylori)* was isolated in 1983s for the first time by Marshall and Warren in Australia (Marshall and Warren, 1984). It is gram-negative, a spiral-shaped microaerophilic bacterium, colonize the human stomach, and infects more than 50% of the world's population (Ahuja and Sharma, 2002; Dunn et al., 1997; Kuipers et al., 1995). Since 1994, it has been recognized as a class I carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization (IARC, 1994).

Typing of *H. pylori*, causing gastritis (G), peptic ulcers (PU) and gastric carcinoma (GC) is crucial for understanding the pathogenesis, treatment and epidemiology (Atherton, 1997; De Luca and Iaquinto, 2004). The typing methods are grouped as phenotypic and genotypic methods (Struelens et al., 1996): phenotypic methods are based on expressed

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characteristics such as antimicrobial susceptibility, lectin typing, biotyping and serotyping. These methods are laborious, time consuming, not widely used and have little discriminatory power as compared to genotypic methods (Hirmo et al., 1997).

Several genotyping methods have recently been developed, including restriction enzyme analysis (REA) of the whole genomic DNA (Owen et al., 1990; Taylor et al., 1995), ribotyping (Lopez et al., 1993), pulsed-field gel electrophoresis (PFGE) (Takahami et al., 1994), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis (Taylor et al., 1995), repetitive extragenic palindromic-polymerase chain reaction (REP-PCR) (van Doorn et al., 1998) and randomly amplified polymorphic DNA (RAPD) (Akopyants et al., 1995). Among them, the PCR-based RFLP analysis has been widely used for typing and differentiation of *H. pylori* strains from clinical isolates (Akopyanz et al., 1992; Moore et al., 1993; Fujimoto et al., 1994).

The aim of this study was to investigate the genetic differences between *H. pylori* isolates obtained from patients with G and PU in Elazig Province, East of Turkey and also to evaluate the relationship among the common RFLP types in patients with G and DU.

MATERIALS AND METHODS

Patients

Sixty one *H. pylori* isolates were obtained from the antrum of Turkish patients (29 males and 32 females) with 20 to 80 years of age (average 47). who attend the endoscopy at Firat University Hospital, Gastroenterology Department during 2009 and 2010. The biopsy specimens for histology fixed in 10% buffered formalin, then embedded in paraffin. Sections in 5 microns thick were stained by hematoxylin and eosin. The grades of G were assessed according to the Sydney classification system by a pathologist (Dixon, 1996).

This study obtained the approval of the Medical Ethics Committee of Firat University and all patients gave informed consent prior to specimen collection. The severity of diseases was diagnosed by pathology and endoscopic findings. Patients were classified in three groups based on clinical outcome as G in 51 cases, PU [duodenal ulcer (DU) or gastric ulcer (GU)], in 10 cases at the time of endoscopy.

Culture of *H. pylori*

The gastric biopsy specimens were transported in Brain Heart Infusion broth (Oxoid, Basingstoke, UK) containing 1.5% glycerol under cool conditions (at 4°C) within 2 h *Helicobacter pylori* isolates were isolated as described by Chomvarin et al. (2006). The suspected colonies were Gram-stained and examined for urease, oxidase, and catalase activities (Goodwin and Wesley, 1993). The isolates were stored at -80°C in Brain Heart Infusion broth containing 15% glycerol until used for *H. pylori* genotyping.

The reference *H. pylori* strains (clinical strains) were kindly provided by Dr. Vildan CANER from Pamukkale University, School of Medicine, Department of Medical Biology, Denizli, Turkey.

DNA extraction

DNA was extracted from *H. pylori* by QIAamp DNA mini kit (QIAGEN, Lot No: 11872534, Cat No: 51306) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until it was used for PCR.

Polymerase chain reaction-restriction-fragment length polymorphism analysis

Isolates confirmed as *H. pylori* positive in PCR were analysed by PCR-RFLP based on *ure*C gene. Amplification was performed in a reaction mixture (50 µl final volume) containing 25 µl 2XPCR Master Mix (Fermentas, K01071), 15 µl distilled water, 1 µM forward and reverse primers (*ure*C-U 5′-AAG AAG TCA AAA ACG CCC CAA AAC-3′, *ure*C-L 5′-CTT ATC CCC ATG CAC GAT ATT CCC-3′) (Li et al., 1997) and 5 µl of genomic DNA. Reaction was performed in Touchdown Thermal Cycler (Hybaid, England). This mixture was subjected to PCR with an inital denaturation step at 94°C for 5 min, followed by 45 cycles at 94°C for 45 s, 59°C for 30 s and 72°C for 1 min 30 s followed by a final extension step at 72°C for 10 min (Andreson et al., 2007). The PCR products were analysed on 1.5% agarose gel electrophoresis, gels were stained with ethidium bromide and visualised by UV illumination. Reference *H. pylori* strains were used in all PCR tests as positive controls and distilled water as negative control.

The amplicons were digested with the restriction enzyme *Hha*I (Fermentas AB, Lithuania) with the recommended Tango buffer according to the manufacturer's instructions and incubated for 16 h at 37°C. The *Hha*I-digested products were separated by gel electrophoresis using 2% agarose and visualized with ethidium bromide (0.5 µg/ml) staining using UV light. A 100-bp DNA ladder (Vivantis) was used to determine the size of bands.

Statistically analysis

The χ^2 test was performed to compare the relationship between the RFLP types and clinical/histopathological outcomes. A p value of <0.05 was considered as statistically significant.

RESULTS

Culture and histopathological results

Helicobacter pylori was isolated from 40, 9, 2, 8 and 2 patients with chronic active, chronic active non-atrophic, chronic atrophic G, DU and GU, respectively.

Polymerase chain reaction-restriction fragment length polymorphism analysis results

All *H. pylori*-positive isolates yielded the expected product size (1.169-bp) for the amplified *ure*C gene. PCR products digested with restriction enzyme *Hha*I, five (A-E) distinct RFLP pattern were obtained. Results of the PCR-RFLP analysis obtained from this study showed that genetic heterogenecity among isolates of *H. pylori* is low.

Table 1. Results of RFLP typing in *H. pylori* isolates obtained from patients with G and PU.

Band profiles	The number of *H. pylori* isolates (%)					
	G			PU		
	Chronic active G (n) (%)	Chronic active non-atrophic G (n) (%)	Chronic atrophic G (n) (%)	DU (n) (%)	GU (n) (%)	Total (n) (%)
A	11 (27.5)	1 (11.1)	-	-	-	12 (19.7)
B	22 (55)	8 (88.9)	2 (100)	2 (25)	-	34 (55.7)
C	2 (5)	-	-	-	-	2 (3.3)
D	4 (10)	-	-	6 (75)	2 (100)	12 (19.7)
E	1 (2.5)	-	-	-	-	1 (1.6)
Total	40	9	2	8	2	61

n: number of *H. pylori* isolates.

Clinical/histopathological correlation between RFLP types and gastrointestinal disease

The most predominant type of *H. pylori* isolates from patients with chronic active, chronic active non-atrophic and chronic atrophic G was type B (55, 88.9 and 100%, respectively) while most of the isolates from patients with DU and GU were type D (75 and 100%, respectively). The remaining two types (C, E) appeared in patients with only chronic active G. In addition, type A (27.5 and 11.1%, respectively) observed in patients with chronic active and chronic active non-atrophic G. The most predominant types were B (55.7%), A (19.7%) and D (19.7%), while C (3.3%) and E (1.6%) types were rare (Table 1).

DISCUSSION

Recently, the prevalence of *H. pylori* infection has become increasingly widespread in Turkey. However, there is very little information on the epidemiology of this bacterium. Thus, more molecular epidemiological studies on *H. pylori* isolates and determining the dominant genotypes among isolates would detect potential risk and aid in the development of improved treatment and control strategies.

The present study aimed at determining the genetic variability of *H. pylori* isolates by the enzyme *Hha*I digestion of a 1.169-bp PCR amplified product of *H. pylori ure*C gene (Li et al., 1997). The discovery of high genomic variability in *H. pylori* offers simple, accurate and efficient molecular typing techniques which are useful in both epidemiological and clinical studies (Ge and Taylor, 1998). PCR-RFLP technique has shown numerous and distinct patterns that can differentiate many strains present in the diverse pathologies caused by this bacterium (Mobley, 1996). The common use of this method not only isolate *H. pylori* infecting the patient, but also direct the first eradication therapy or evaluation of the outcome of previous eradication regimens of the patient (with a consequent reduction in cost) (Kargar et al., 2011).

One of the main proteins of *H. pylori* is urease and the genes encoding this and its accessory proteins including the 2.4-kb *ure*A-*ure*B, 1.7-kb *ure*C-*ure*D (Forman et al., 1991; Akopyanz et al.,1992), 933-bp *ure*B (Clayton et al., 1993) and 820-bp *ure*C (Fujimoto et al., 1994) genes have been studied for PCR because these genes are highly conserved in *H. pylori* (Fujimoto et al., 1994; Owen et al., 1998; Burucoa et al., 1999).

Various restriction enzymes have been commonly used for the RFLP analysis of *H. pylori* strains, such as *Hae*III, *Sau*3A, *Hha*I, *Mbo*I, *Hind*III, *Alu*I, *Pvu*I or *Mse*I (Clayton et al., 1993; Moore et al., 1993; Fujimoto et al., 1994; Taylor et al., 1995; Shortridge et al., 1997; Hartzen et al., 1997).

In our attempt to evaluate the genetic differences among *H. pylori* isolates, a 1.169-bp fragment of *H. pylori ure*C gene was amplified by PCR. In the present study, all tested isolates generated a PCR product of expected size (1.169-bp). Five different band profiles were yielded after the examination of *H. pylori* isolates with RFLP analysis using *Hha*I restriction enzyme and these isolates could be classified into five types: A, B,

C, D, and E. Type B showed the highest rate in patients with chronic active, chronic active non-atrophic and chronic atrophic G. In contrast, most of *H. pylori* isolates from patients with DU and GU were type D. The remaining two types (C, E) appeared in patients with only chronic active G. In addition, type A observed in patients with chronic active and chronic active non-atrophic G. Our data supports the findings of Moore et al. (1993), Lin et al. (1998) and Gzyl et al. (1999), who reported that the pathogenicity of *H. pylori* isolates was associated to the RFLP type but in contrast to a study demonstrated that the RFLP type of the *ureC* gene is not related to ulcers, G and portal hypertension (Mishra et al., 2002).

Incomparison with the data from our study, most typing studies related to PCR-based RFLP analysis of *H. pylori* isolates have determined much higher genetic diversity (Fujimoto et al., 1994; Chuanfu et al., 1997; Stone et al., 1997; Tanahashi et al., 2000; Colding et al., 2003; Röesler et al., 2009; Agudo et al., 2011). However, the use of different primers and more than one enzymes or other typing methods may be needed to improve the differentiation power of PCR-RFLP. Reason of less PCR-RFLP profiles obtained in our study than other studies may be due to that only one enzyme is used. However, different results obtained by PCR-RFLP typing may be due to geographic polymorphism in the distribution of *H. pylori* isolates.

This study reports on PCR-RFLP analysis of *H. pylori* isolates in Elazig Province East of Turkey for the first time and provides important data for further studies on this subject. However, more detailed molecular epidemiological studies are needed to compare *H. pylori* isolates from other geographical regions to help us understand better the epidemiological importance of this disease.

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Abbreviations: *H. pylori*, *Helicobacter pylori*; **G,** gastritis; **PU,** peptic ulcer; **DU,** duodenal ulcer; **GU,** gastric ulcer; **GC,** gastric carcinoma; **IARC,** ınternational agency for research on cancer; **PCR,** polymerase chain reaction; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **REP-PCR,** repetitive extragenic palindromic-polymerase chain reaction; **RAPD,** randomly amplified polymorphic DNA.

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