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Annals of Biological Research, 2011, 2 (1) : 32-39
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0976-1233
CODEN (USA): ABRNBW

Helicobacter pylori infection and colorectal cancer in Guilan province of Iran

Zivar Salehi[1], Mohammadamin Miri[1], Keyvan Aminian[2] and Fariborz Mansour-Ghanaei[2]

[1]Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran
[2]Gastrointestinal and Liver Diseases Research Center (GLDRC), Guilan University of Medical Sciences (GUMS), Rasht, Iran

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ABSTRACT

Infection of the gastric mucosa with Helicobacter pylori (H. pylori) results in a number of disease outcomes including gastritis, which precedes the development of peptic ulcer disease, gastric cancer, and lymphomas of the mucosa associated lymphoid tissue lymphoma. The aim of this study was to assess association of colorectal cancer with H. pylori infection in Guilan province of Iran. Fifty-eight patients with colorectal cancer were enrolled in our study. Specific polymerase chain reaction assays were used for three genes: glmM, vacA and cagA. Of the 20 glmM PCR-positive bacterial colonies, 15 (75%) had the vacA signal sequence genotype s1, and 5 (25%) had subtype s2. The vacA mid- region analysis revealed that 13 (65%) were vacA m1 and 7 (35%) were m2. The cagA gene was present in 13 (60%) of colorectal cancer specimens. These results show that H. pylori can be detected in the tumor tissues specimens of some patients with colorectal cancer by polymerase chain reaction (PCR). vacA s1 is important virulence determinant of H. pylori in patients with colorectal cancer.Further studies are necessary to assess the potential role of H. pylori in the development of colorectal cancer.

Key words: H. pylori, colorectal cancer, cagA, vacA, glmM.

INTRODUCTION

Colorectal cancer is the fourth most common cancer in Iran [1]. Epidemiological studies have suggested that colon cancer is a manifestation of a number of inherited cancer predisposition syndromes, including familial adenomatous polyposis, hereditary non-polyposis colorectal cancer, and personal or family history of colorectal cancer and/or polyps and inflammatory bowel disease [2]. Furthermore, other factors such as obesity, lack of exercise, smoking, alcohol consumption, diet rich in high fat, red and processed meats and inadequate intake of dietary fiber, fruits and vegetables are also associated with increased colon cancer risk [3-5].

Helicobacter pylori infection has been associated with an increased risk for colorectal neoplasia in some studies [6, 7]. However, the results of these studies remain controversial.

Helicobacter pylori is a spiral-shaped gram-negative bacterium and was identified as the cause of chronic active gastritis, peptic ulcer disease [PUD), and is considered to be a risk factor for the development of gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma [8, 9]. The clinical outcome of *H. pylori* induced pathology is likely to be determined by a combination of factors including the pathogen virulence factors, host immune responses, and capacity for colonizing new host niches [10]. The vacuolating cytotoxin VacA [11] and the *cagA* pathogenicity island [12] are two identified virulence factors that are considered to have an important role in the pathogenesis of *H. pylori* infection. VacA, which *in vitro* induces vacuolation in epithelial cells, is encoded by *vacA*, which has distinct allelic types [13]. The *vacA* genotype is determined by a combination of two main regions within the gene: a midregion (m region) and the signal sequence (s region). *H. pylori* strains with s1/m1 or s1/m2 *vacA* gene subtypes were able to produce high or moderate levels of VacA, respectively, whereas strains with s2/m2 subtype were not. The *vacA* s1/m1 genotype is thought to be associated with more severe pathologies [11- 13].

The *cag* pathogenicity island (*cagA*) is one of the major virulence determinants of *H. pylori*. It encodes a type IV secretion system (T4SS), a multimolecular complex that mediates the translocation of bacterial factors into the host cell [8]. The *cagA* gene encodes the CagA protein and has been used as a marker for the presence of the *cag PAI*. Persons colonized with *cagA*-positive strains are at increased risk for developing peptic ulceration and distal gastric cancer compared to person harboring *cagA*-negative strains [14].

The aim of this study was to investigate if *Helicobacter pylori* DNA could be detected in colorectal cancer biopsy specimens. Since considerable geographic diversity in the prevalence of *H. pylori* virulence factors has been reported, we have also investigated the *vacA* genotypes and *cagA* status in *H. pylori* positive samples.

MATERIALS AND METHODS

Subjects

Fifty-eight patients with colorectal cancer referred at the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Sciences, Iran, during December 2008 and November 2009 were recruited into the study. Colonoscopy was performed with standard colonoscop (Olympus, Japan). The specimens were obtained by using sterilized biopsy forceps cleansed with a detergent, disinfected with 70% ethanol, and rinsed with sterile water after each examination. Different forceps were used for each biopsy. One biopsy specimens was obtained from the mucosa at a distance of 50 cm from the tumor as a control. The institutional ethics committee approved the study protocol and all the patients gave informed consent to participate in the study. In addition, detailed information on clinical features, including the exact location, stage, and histology of the tumors, was obtained from hospital charts and pathology reports. Patients taking antibiotics, proton pump inhibitors and/or non-steroidal anti-inflammatory drugs in the preceding month, with a history of chemotherapy or radiation therapy before surgery were excluded from the study.

Culture of *H. pylori* from biopsy specimens

Colorectal biopsy specimens obtained from of all patients and were cultured as described previously [15]. Briefly, specimens placed in 1 ml of normal saline (0.9% sodium chloride)

were dissected and spread on Skirrow agar containing 5% horse blood and Skirrow's antibiotic supplement under microaerobic conditions in an anaerobic jar at 37ºC for a minimum of 7 days. The cultured bacteria were defined to be *H. pylori* if they formed typical colonies on the medium, were negative Gram stain with curved or spiral shape, and positive for urease and catalase production [16].

Molecular detection of *H. pylori*

The presence of *H. pylori* DNA was confirmed using PCR amplification of the phosphoglucosamine mutase gene, *glmM* (*ureC*). The oligonucleotide primers were synthesized in an automated DNA synthesizer and were purified with high-performed liquid chromatography. Primers GLMF and GLMR amplified 294 bp product from the *glmM* gene. PCR reaction was carried out as described [15].

***vacA* genotypes**

Primers VA1-F and VA1-R have been described [13] and generate a fragment of 259 bp for s1 variants and a fragment of 286 bp for the s2 variants. For analysis of mid region (m region), PCR was performed with VA3-F, VA3-R VA4-F and VA4-R. These primers generate a fragment of 290 bp for m1 variants and a fragment of 352 bp for m2.

***cagA* status**

PCR amplification of *cagA* was carried out with primers CagA/ConF and CagA/ConR. PCR reaction was carried out as described [14]. These primers generate a fragment of 402 bp. A 10 µl of PCR product was then analyzed by electrophoresis on 2% agarose gel run in TBE buffer and stained with ethidium bromide. The PCR product was examined in parallel with 100 bp DNA ladder.

Statistical Analysis

Statistical analysis was performed using the χ^2 test (Web Chi Square Calculator; http://hg.wustl.edu/info/linkage/web-chi/web-chi-form5.htm) and the Med Calc version 9.3. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Subjects

The general characteristics of the cases are shown in Table 1. The most common manifestations were anemia and rectal bleeding. There were 40 (%69) men and 18 (%31) women. As expected, most colorectal cancer patients were aged over 50 years. The mean age was 58.7 ± 4.6 years (range 43- 72 years). The cancer was found in the rectum of 23 patients (%39.6), in sigmoid of 20 patients (%34.5), in transverse colon of 9 subjects (%15.5), in descending colon of 2 patients (%3.4) and in ascending colon of 4 patients (%6.9).

Histopathology

Sections of biopsy specimens were examined without knowledge of the experimental PCR results by one experienced histopathologist. All pathological samples were classified in accordance with the American joint committee on cancer [17]. Only 20.6 % and 29 % of the cases were diagnosed at stage I and stage III, respectively, whereas 50% of the cases were diagnosed with a stage II cancer.

Prevalence of *H. pylori* infection

By using primers GLMF and GLMR to amplify the *glmM* gene, the expected PCR product of 294 bp was obtained in 20 (34.5%) patients (Figure 1). The Fifty-eight normal mucosal specimens were all negative for *H. pylori* DNA.

Determination of *vacA* genotypes

All *H.* pylori positive specimens were positive for the *vacA* gene, as evidenced by PCR product sizes, which enabled to differentiate *s* and *m* alleles (Figures 2, 3). Among the samples, 15 (75%) were found to have *vacA* s1, followed by s2 (5, 25%). In the m-region, m1 was most commonly found (13, 65%) followed by m2 (7, 35%). The *vacA* genotype combinations were as follows: s1/m1, n=10; s1/m2, n= 5; s2/m1, n=3; s2/m2, n= 2 (Figure 4).

Detection of *cagA* in colonic biopsy specimens

Of the 20 *glmM* PCR-positive biopsy specimens, 13 (60%) also gave amplified DNA fragments in *cagA* PCR. Figure 5 shows that when the *cagA* gene was amplified, a 402-bp PCR product was visualized as a unique and homogenous band.

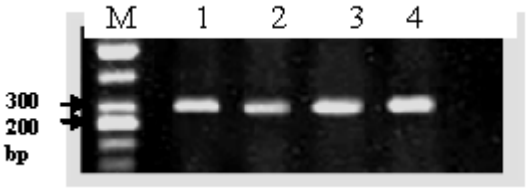

Figure 1. Agarose gel electrophoresis of *glmM* PCR product obtained from the clinical isolates of four patients using the primer pair glmMF/glmR (Lanes 1-4, 294 bp amplicon). Lane M, molecular size marker.

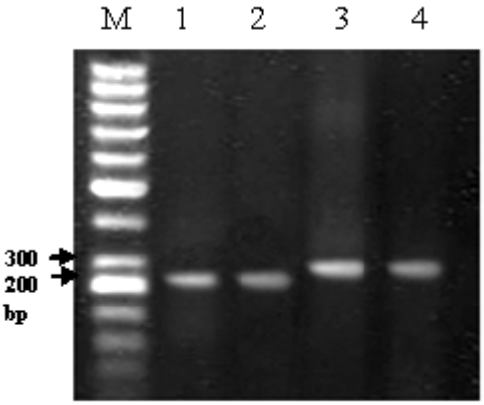

Figure 2. Detection of s1 and s2 alleles. Lane M, DNA marker; lanes 1, 2, s1 allele (259 bp); lanes 3, 4, s2 allele (286 bp).

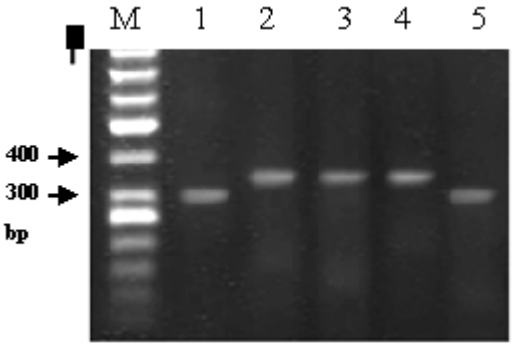

Figure 3. Detection of m1 and m2 alleles. Lanes 1, 5, m1 allele (290 bp); lanes 2, 3 and 4, m2 allele (352 bp). The molecular weight marker is shown in the left part of the gel.

Relationship between *cagA* status and *vacA* genotypes

After *H. pylori* genotyping, 7 (40%) were classified as infected with *cag-A* negative *H. pylori*, 13 as infected with *cagA*-positive *H. pylori* and 38 as uninfected. The genotype s1/m1 *cagA*-positive was the most frequent among patients with colorectal cancer (16/20, $P<0.001$).

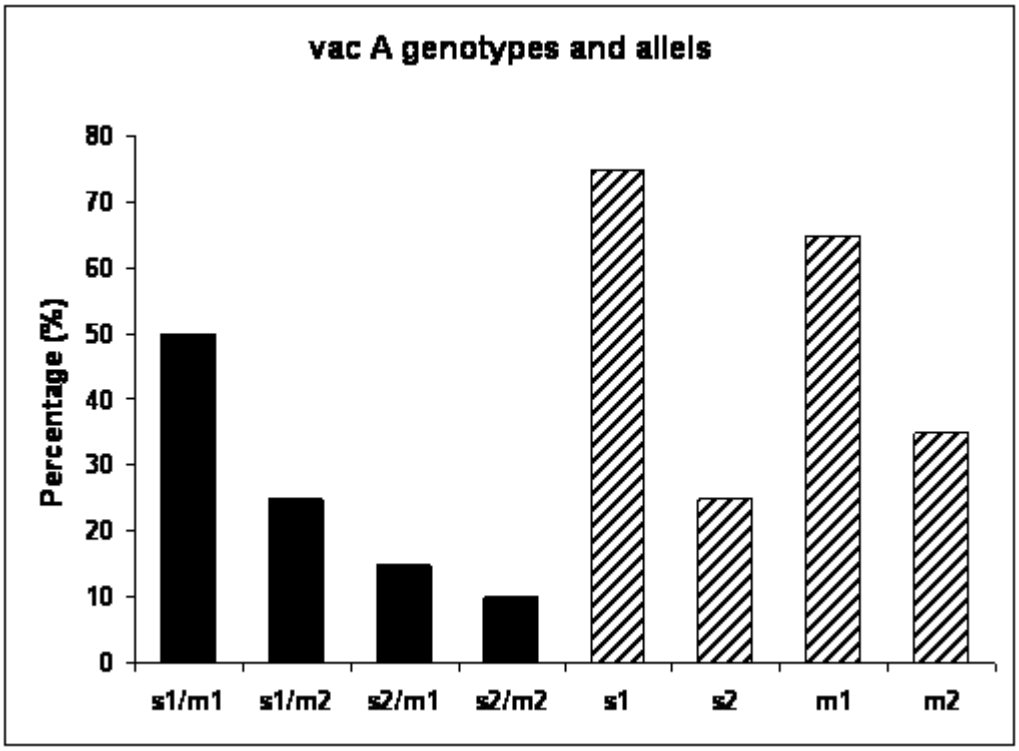

Figure 4. Distribution of *vacA* genotypes and alleles in patients with colorectal cancer.

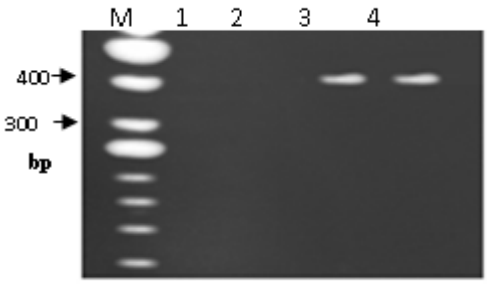

Figure 5. Agarose gel electrophoresis after PCR amplification of *cagA* gene. Lanes: M, molecular marker; 1 and 2, *cagA* negative; 3 and 4, *cagA* positive.

DISCUSSION

Helicobacter pylori infection of the human stomach is the most important risk factor for development of gastric cancer. The exact mechanisms that lead to cancer induction are not clear, but study of the bacterial factors important for colonization and the host responses to the infection are starting to yield important clues. *H. pylori* infection has been associated with an increased risk for colorectal neoplasia in some studies. However, the results of these studies remain controversial. In Iran, the role of *H. pylori* infection has been implicated in peptic ulcer disease, but the occurrence of *H. pylori* infection has not been examined in colorectal cancer, which is one of the most common causes of cancer-related death in Iran[1].

The prevalence of *H. pylori* infection is about 50% of the world's population and has been reported to be 60-90% in Iran [15, 18].

In this study, the *glmM* gene was used for detection of *H. pylori* in biopsy samples, using PCR. We detected the *H. pylori* genomic DNA in 20 (34.5%) tumor tissues but not in normal colonic mucosa. There is a significant correlation between the presence of *H. pylori* and colorectal cancer (odds ratio= 30.52; 95%CI= 3.93-237.03; $P<0.005$). Numerous *Helicobacter* spp. have also been isolated from the intestinal tracts of humans, animals, and birds. Of particular interest are *Helicobacter bilis* and *Helicobacter hepaticus* because of their association with hepatitis and inflammatory bowel disease in several strains of mice [19, 20]. In an investigation for *H. pylori* 16S rDNA with PCR in colon cancer biopsies, Grahn et al. revealed *H. pylori* in 27% of cancer tissue specimens [8]. However, in study conducted in 83 patients with colorectal cancer, the overall prevalence of *H. pylori* infection, assessed by PCR in biopsy specimens from tumor tissue, was 1.2% [7].

Our results also demonstrate an association between the *vacA* s1 allele and the presence of colorectal cancer. It has been reported that *H. pylori* isolates with *vacA* s2 fails to induce cell vacuolation *in vitro* [13]. It has been shown that s1 allele was related to peptic ulcer disease such as duodenal and gastric ulcer [21]. In addition, we have previously shown that the *vacA* s1 is the most common allele in Iranian patients with PUD [22].

Gastric carriage of *H. pylori* particularly *cagA* positive strains, is known to be a risk factor for peptic ulcer disease and gastric cancer and may have a similar etiologic relationship with colon cancer. Although *H. pylori* infection occurs worldwide, there are significant differences in its prevalence both within and between countries [23]. The *cagA* positivity in Iranian isolates has been reported to vary from 44% to 91% in different reports [15, 24, 25]. We found that 13 (60%) of *H. pylori* positive samples amplified *cagA* constant region. The presence of *cagA* gene showed a strong correlation with colorectal cancer (odds ratio=105.85; 95% CI=11.96-936.63; $P<0.0001$). Three serologic studies have shown that infection with *H. pylori* significantly increases risk for development of colon cancer. Shmuely et al. reported that *H. pylori* CagA+ seropositivity is enhanced in gastric and colon cancer (odds ratio = 10.6; 95% CI = 2.7-41.3; P = 0.001) [26], while Fireman et al. demonstrated a correlation between *H. pylori* seropositivity and CA19-9 elevation in patients with colon cancer [27]. In the evaluation by Mizuno et al. of the colon pathologies of 332 patients with high-resolution colonoscopy, the increase in the incidence of adenomatous polyps in *H. pylori* IgG seropositive patients and diminution of normal colonoscopy findings was found more significant than in seronegative patients [28].

The pathogenic mechanisms by which *H. pylori* exerts its malignant potential are unknown. However, gastrin might be a key factor triggered colorectal tumor development [29]. A number of studies have shown that *H. pylori* infection may induce hypergastrinemia in patients with duodenal ulcers [30, 31]. Hypergastrinemia may be involved in the increased risk of colorectal tumor development in *H. pylori*-infected subjects [32]. Additional factors, including intestinal flora, ammonia levels, vacuolating toxin VacA and CagA could also lead to increased risk of colorectal cancer [33, 34].

CONCLUSION

In conclusion, this pilot study carried out in Iran focused on 58 patients with colorectal cancer. A strong association between *H. pylori* infection and colorectal cancer was confirmed

overall. The presence of the *cagA* gene correlated with *vacA* signal type s1. Further studies with larger sample size, host genetic susceptibility, bacterial variants and dietary habits and different types of colorectal neoplasm are needed to examine the role of *H. pylori* in colorectal carcinogenesis.

Acknowledgements
The authors would like to thank everyone at the Molecular Genetics Laboratory, Department of Biology, University of Guilan, Rasht, Iran, and Department of Endoscopy, Razi Hospital, Rasht, Iran for their help.

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