



Mutation in *KRAS* and *BRAF* Genes in *Helicobacter pylori*-Infected Patients with Gastric Cancer and Peptic Ulcer

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Abstract

Background: *Helicobacter pylori* are the main cause of various gastroduodenal diseases. It is estimated that approximately half of the planet population is infected with *H. pylori*. *KRAS* and *BRAF* genes are the targets of genetic changes in *H. pylori*-infected patients with gastric cancer (GC) and peptic ulcer (PU). The high frequency of these mutations represents their high potential as a biomarker in early diagnosis of GC.

Objectives: The current study aimed at evaluating the frequency of *KRAS* (Kirsten Rat Sarcoma) and *BRAF* (*BRAF* proto-oncogene) gene mutation status in *H. pylori*-infected patients with GC and PU.

Methods: The current cross-sectional and descriptive study was conducted on 80 paraffin-embedded sections including 40 gastric adenocarcinoma and 40 PU tissue samples. The samples were collected from April 2017 to March 2018. *H. pylori* were identified and confirmed in all samples using the IHC (immunohistochemical) method and the histopathology of all PU and GC tissue samples was available. After DNA extraction from paraffin-embedded sections, and polymerase chain reaction, *KRAS* and *BRAF* gene mutations were assessed using the direct sequencing method, and the correlation of mutations and clinicopathological characteristics was also studied.

Results: *KRAS* mutation was observed in codon 12 (n = 7; 17.5%) and *BRAF* mutation in codon V600 (n = 4; 10%) in patients with GC. No *KRAS* and *BRAF* mutations were observed in patients with PU. Results of the current study also showed that the majority of the examined samples belonged to male patients (70%) and female patients constituted 30% of the samples; patients mean age was 48.95 ± 12.11 years. No significant correlation was observed between the mutations and pathological manifestations (age, gender, and tumor grade).

Conclusions: *KRAS* and *BRAF* gene mutations were revealed in *H. pylori*-infected patients with GC.

Keywords: Adenocarcinoma, *BRAF*, Gastric Cancer, Gene, *Helicobacter pylori*, *KRAS*, Mutation, Neoplasm, Peptic Ulcer, Stomach

1. Background

Helicobacter pylori are curved rod, Gram-negative, microaerophilic, highly motile bacteria that cause infection in the gastric mucosa, especially in the pyloric antrum (1). *H. pylori* are successful pathogens that adhere to gastric epithelial cells via different adhesins and use their virulence factors to cause the disease. It is the most important cause of gastrointestinal (GI) diseases including chronic gastritis, duodenal ulcer, and gastric cancer (2). The World Health Organization (WHO) identified *H. pylori* as a group 1 carcinogen. Among diseases caused by these bacteria, gastric cancer (GC) is of great importance (3). *H. pylori* are

very sensitive to gastric acid, and therefore, use their flagella to burrow into the mucus lining of the stomach. They produce urease to neutralize the acidic area surrounding the bacteria to facilitate movement towards the gastric mucosa. After adherence to epithelial cells, they colonize mucosa, ultimately resulting in ulceration (4). The interaction between *H. pylori* and gastric tissue leads to cellular changes; these bacteria can adhere to laminin and induce apoptosis of the epithelial cells, resulting in activation of oncogenic pathways in host cells (5). *KRAS* is one of the most important molecules in the signaling downstream of EGFR (epidermal growth factor receptor). *KRAS* is a proto-oncogene that encodes a 21-kDa GDP/GTP bind-

ing protein. It is located on the short arm of chromosome 12 and has a critical role in the transduction of mitogenic signals and regulation of cellular responses to extracellular stimuli such as growth factor, cytokines, and hormones (6, 7). Similar to other G proteins, the GDP-bound form is inactivated, and the GTP-bound form is activated. *KRAS* mutations result in the deactivation of GTPase and activation of the *RAS/RAF* signaling pathway. *RAS* gene mutations are responsible for many human cancers and found in 17% - 25% of all GC and carcinoma cases (8, 9). *BRAF* proteins are a member of the serine/threonine-protein kinase *RAF* family. Mutant *BRAF* proteins enhance kinase activity and can transform NIH₃T₃ cells. Mutation in this gene causes resistance to anti-EGFR therapy in patients with cancer (10). *KRAS* and *BRAF* mutations are common in different cancers and play an important role in tumor progression, metastasis, and response to treatment in patients with cancer. Therefore, identification of the mutation in these genes is essential in cancer studies (11). Since *BRAF* and *KRAS* mutations can be detected in early cancer stages, they can be used to identify people who are at risk for GC to increase their survival (12). *KRAS* mutations are point mutations in exon 12 and 13, and *BRAF* mutations are located at codon 599/600 in more than 90% of the cases (13). Molecular and genetic markers in *H. pylori*-infected patients with GC are affected by environmental and genetic factors as well as the amount of exposure to mutagens (14). Epidemiological studies in Iran show that the incidence of GC in Iran is higher than those of other countries. Moreover, it is proved that molecular pathways involved in GC such as mutations, polymorphisms, protein expression, and *H. pylori* as a carcinogen, affect disease progression and respond to treatment (15).

2. Objectives

The current study aimed at concomitantly evaluating the status of *KRAS* and *BRAF* mutations in *H. pylori*-infected GC and peptic ulcer (PU) tissue samples of 80 patients by polymerase chain reaction (PCR) and directed sequencing. In addition, these variants are usually evaluated in order to aid the selection of patients for treatment and the prognosis of GC.

3. Methods

3.1. Study Population

The current descriptive cross-sectional, case-control study was conducted to evaluate mutations in exon 2 and hot-spot codon 12 of the *KRAS* gene and hot-spot codon V600 in exon 15 of the *BRAF* gene in *H. pylori*-infected gastric

adenocarcinoma and *H. pylori*-infected PU tissue samples. The inclusion criteria were: fulfillment of the informed consent, age 27-74 years, complaining of upper abdominal pain, nausea, and bloating for two months, and confirmed the histological diagnosis of *H. pylori*-infected GC and *H. pylori*-infected PU. The exclusion criteria were patients with non-viable tissue blocks (inadequately fixed/processed), use of antibiotics in last month, and negative results for *H. pylori* test. The sample size was calculated based on the study by Yang et al. (16), with a 95% confidence interval and a test power of 80%. In the current study, a total of 342 patients were enrolled, of which 178 had GC and 155 PU. Each subject was evaluated for *H. pylori* infection, and 44 out of 178 patients with GC were positive for *H. pylori* infection. For the control group, 44 subjects with positive *H. pylori* were selected out of 155 patients with PU. After DNA extraction, four samples from patients with GC were unsuitable for sequencing and were excluded; therefore, the final sample size was 40 subjects; 80 paraffin-embedded tissue samples belonging to 40 *H. pylori*-infected patients with GC and 40 *H. pylori*-infected patients with PU referring to 5th Azar Hospital, Gorgan, Iran from April 2017 to March 2018, with a mean age of 48.95 ± 12.11 years (range: 27 - 74) were assessed. The current study protocol was approved by the Ethics Committee of Ayatollah Amoli branch, Islamic Azad University (ethical code: IR.IAU.AMOL.REC.23930507971001). Written informed consent was obtained from all subjects included in the study, and all experiments were conducted in accordance with the Declaration of Helsinki. The biopsy samples were taken from the pyloric antrum by a gastroenterologist and then, were fixed in 10% formalin and cut into 5-6- μ m thick sections by a microtome. The presence of *H. pylori* in all samples was assessed by urease test, hematoxylin and eosin (H&E) staining, Giemsa staining, and IHC (immunohistochemical) method. To detect *H. pylori*, IHC was performed using *H. pylori* antibody (DAKO, High Wycombe, UK) according to the manufacturer's protocol (Figure 1) (17). The clinicopathological features of all patients were verified by experienced pathologists (Table 2).

3.2. DNA Extraction

Genomic DNA was extracted from tissue samples by a standard method provided by Qiagen kit (Germany) according to the manufacturer's instructions (18).

3.3. PCR Amplification and Sequencing

KRAS (codon 12) and *BRAF* (codon 600) were amplified using PCR with related primers. The PCR was run in a thermalcycler (Eppendorf Mastercycler Gradient, Eppendorf, Hamburg, Germany). Table 1 shows the sequences

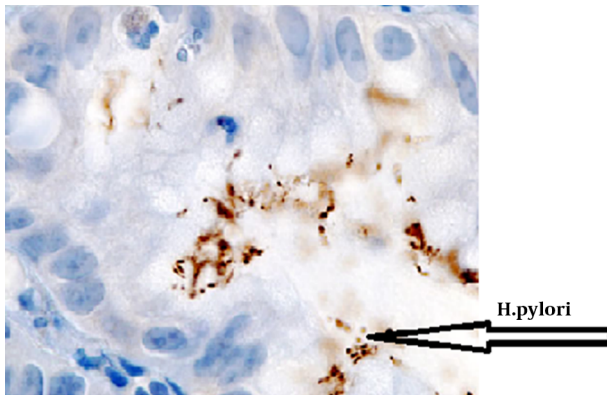


Figure 1. Immunohistochemical staining of *H. pylori*

Table 1. Primer Sequences of *KRAS* and *BRAF* Genes

Gene	Primer Sequences	Reference
<i>KRAS</i>		(19)
Forward	5'-AAAATGACTGAATATAAACTTGTGG-3'	
Reverse	5'-CICATATTGTTGGATCATATTCGTC-3'	
<i>BRAF</i>		(19)
Forward	5'-CTTCATGAAGACCTCACAGT-3'	
Reverse	5'-CATCCACAAAATGGATCCAG-3'	

of primers related to the *KRAS* and *BRAF* genes used in the PCR after blasting in NCBI GenBank. The program used to amplify both *KRAS* and *BRAF* genes was as follows: Denaturation at 95°C for five minutes followed by 40 cycles at 95°C for 30 seconds, 62°C for 30 seconds and 72°C for one minute. PCR products were electrophoresed on a 1.5% agarose gel. Sequencing of PCR products was performed by MacroGen Company and the results were compared. The sequences were analyzed on ABI 3730XL (Applied Biosystem). The resulting sequences were analyzed using Chromas software. Each mutation was verified in the sense direction.

3.4. Statistical Analysis

The statistical analyses were performed using the SPSS Statistics for Windows, version 18.0 (SPSS Inc., Chicago, Ill., USA). The descriptive statistics were employed to calculate frequencies and percentages for clinicopathological features such as age, gender, tumor grade, and frequency of mutation. Comparisons between qualitative data were made using chi-square test and $P < 0.05$ was considered statistically significant.

Table 2. Demographic Features of *H. pylori*-Infected Patients with Gastric Cancer or Peptic Ulcer^a

Feature	Group 1 (PU), N = 40	Group 2 (GC), N = 40
Gender		
Male	23 (57.5)	28 (70)
Female	17 (42.5)	12 (30)
Age, y	44.4 ± 9.75	52.3 ± 12.11
Age group, y		
Above 50	11 (27.5)	23 (57.5)
Below 50	29 (72.5)	17 (42.5)
Tumor grade		
1	0	10 (25)
2	0	4 (10)
3	0	11 (27.5)
4	0	15 (37.5)

^a Values are expressed as No. (%) or mean ± SD.

4. Results

The current study was conducted on two groups of patients, 40 patients with GC and 40 patients with PU. *H. pylori* infection was confirmed in all patients using IHC method as well as Giemsa and H&E staining. The demographic data of the study patients are presented in Table 2. Of 40 *H. pylori*-infected patients with GC, 28 (70%) were male and 12 (30%) female. Among 40 *H. pylori*-infected patients with PU, 23 (57.5%) were male and 17 (42.5%) female. The male-to-female ratio was 2:3 in the study. Of the 40 sequenced GC samples, seven patients (17.5%) had mutation in codon 12 of *KRAS* gene, and four patients (10%) had mutation in codon V600 of *BRAF* gene. In addition, six mutations occurred exclusively in male patients aged over 50 years. No *KRAS* and *BRAF* mutations were observed in patients with PU. The results showed a higher frequency of the *KRAS* and *BRAF* mutations in male patients, but there was no significance between gender and the presence of *KRAS* and *BRAF* mutations ($P_{\text{value}_{KRAS}} = 0.872$, $P_{\text{value}_{BRAF}} = 0.530$). The odds of *KRAS* and *BRAF* mutations were higher in male subjects than female ones. The results also showed no significant correlation between mutations in *KRAS* and *BRAF* genes and tumor grade in *H. pylori*-infected patients with GC ($P_{\text{value}} = 0.333$) (Tables 3 and 4).

4.1. *BRAF* Codon 600 and *KRAS* Codon 12 Mutations

BRAF and *KRAS* genes DNA sequencing was performed for PCR products from both studied groups, and it was observed that DNA sequence analysis of *KRAS* (codon 12) had G to A transversion at gastric adenocarcinoma (Figure 2).

Table 3. Associations Between *KRAS* Mutation and Clinicopathological Features in Patients with Gastric Cancer^a

Feature	Patients Positive for <i>KRAS</i>	Patients Negative for <i>KRAS</i>	P Value
Total number	7 (17.5)	33 (82.5)	
Gender			0.872
Male	4 (57.2)	22 (66.6)	
Female	3 (42.8)	11 (33.3)	
Tumor grade			0.333
1	2 (28.5)	8 (24.2)	
2	4 (57.1)	11 (33.3)	
3	0 (0)	11 (33.3)	
4	1 (13.4)	3 (9.2)	

^a Values are expressed as No. (%).**Table 4.** Associations Between *BRAF* Mutation and Clinicopathological Features in Patients with Gastric Cancer^a

Feature	Patients Positive for <i>BRAF</i>	Patients Negative for <i>BRAF</i>	P Value
Total number	4 (10)	36 (90)	
Gender			0.530
Male	3 (75)	21 (58.3)	
Female	1 (25)	15 (41.7)	
Tumor grade			0.492
1	1 (25)	9 (25)	
2	2 (50)	13 (36.1)	
3	0 (0)	11 (30.6)	
4	1 (25)	3 (8.3)	

^a Values are expressed as No. (%).

DNA sequence analysis of *BRAF* (codon V600) had T to A transversion at gastric adenocarcinoma. No *KRAS* and *BRAF* mutations were observed in patients with PU. *KRAS* and *BRAF* mutations had no significant correlation with clinicopathological characteristics.

5. Discussion

In the previous decade, molecular targeted therapy was an important area of investigation to treat human cancer. However, contemporary targeted therapy for GC is less than satisfactory (20). There are inconsistent reports of the prevalence of GC across the world, which could be due to differences in the geographical conditions, diet, and exposure to carcinogens, and lifestyle characteristics. On the other hand, *H. pylori* are gastric carcinogens and their adherence to the gastric antral epithelial cells prepares the grounds for mutations, ultimately resulting in GC (21). The current study aimed at evaluating *KRAS* and *BRAF* mutations in *H. pylori*-infected patients with GC and the importance of designing proper treatment strategies for them. *KRAS*, a molecular switch of intracellular signaling transduction pathways, is essential in transferring extracellular growth signals into the nucleus. The mutations

of *KRAS* and *BRAF* genes were associated with those of numerous types of human cancer including pancreatic cancer, colorectal cancer (CRC), GC, and lung cancer (22). *BRAF*, a member of the *RAF* kinase family, is an essential downstream effector of *KRAS*. *BRAF* is commonly activated by somatic mutation (23). Many researchers believe that *KRAS* and *BRAF* mutations are limited and exclusive to CRC, while Lee et al. conducted a study on *KRAS* and *BRAF* mutations in GC and reported that the genes were frequently mutated in gastric adenocarcinoma and were, therefore, of great importance in this cancer. Similarly, *KRAS* and *BRAF* mutations are observed in *H. pylori*-infected patients with GC (21). The frequency of mutation in the *KRAS* gene in different studies varied markedly. In order to improve understanding of the status of mutations in the *KRAS* gene in GC, a large international multicancer study including 712 patients with GC was conducted in 2013. In this study, which is the largest to date, the frequency of mutations in the *KRAS* gene was 4%, and it was not associated with clinicopathological features, including ethnicity, gender, and grade of the tumor. This finding was consistent with the current study results. In the current study, the most frequent mutation in codon 12 of the *KRAS* and codon V600 of the *BRAF* was a nucleotide change, resulting in coding aspartate instead of glycine. This finding was consistent with the re-

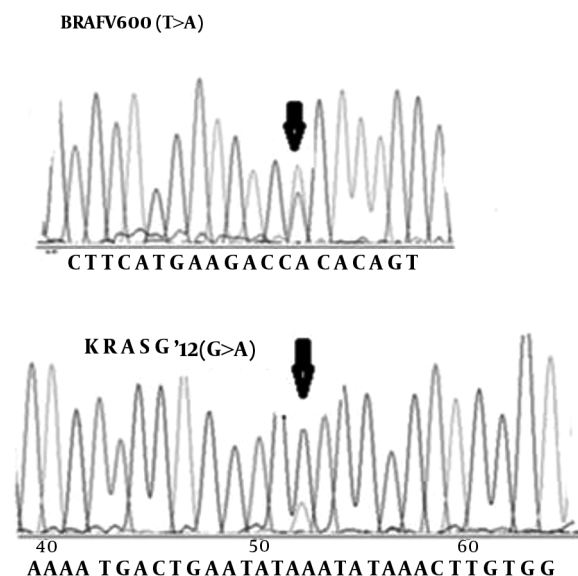


Figure 2. The results of DNA sequence mutation analysis of *KRAS* and *BRAF* oncogenes; DNA sequence analysis of *BRAF* gene (codon v600) had T to A transversion; DNA sequence of *KRAS* gene (codon 12) had G to A transversion in patients with gastric cancer.

sults of a study by Sabry et al. They also reported that *H. pylori* infection was correlated with epigenetic changes, *KRAS*, and *BRAF* mutations, and clinicopathological characteristics (19). In the current study, the presence of *KRAS* and *BRAF* mutations (codon 12 and V600, respectively) was investigated in all 80 patients with GC and PU using the direct sequencing method. Takahashi et al. performed a similar study and reported that the frequency of *KRAS* mutation was 4.9% in patients with GC. Their study indicated that mutations of *KRAS*, *PIK3CA*, and *NRAS* were rare in gastric adenocarcinoma (24). In the current study, the methodology of previous studies was employed to evaluate *BRAF* and *KRAS* mutations in patients with GC and PU using the DNA sequencing method (21). In the current study, 45 patients were below and 23 were above 50 years. According to the results of the current study and other investigations, age could affect the incidence of *H. pylori* infection and its pathological characteristics. The mean age of the patients was 52.3 years in the current study, which is consistent with the results of Mohebbi et al., in their latest report on GI cancers (25, 26). Few studies quantitatively assessed *KRAS* and *BRAF* mutations in gastric adenocarcinoma; therefore, no accurate statistics are available in this regard. The current results confirmed *KRAS* and *BRAF* mutations in *H. pylori*-infected patients with GC. In the present study, all 40 patients with GC were investigated for *BRAF* mutation in

codon V600 using the direct sequencing method, and the results showed 10% mutation in this codon. This finding was consistent with the results of Yang et al. (16). No significant correlation was found between *KRAS* and *BRAF* mutations and the clinicopathological features of the patients, including gender, age, and tumor grade; however, the frequency of *KRAS* and *BRAF* mutations was higher in grades 3 and 4. This relationship was direct, since the odds of *KRAS* and *BRAF* mutations increased with grade increase (grade 4). Several studies confirmed this finding (27, 28). Thus, mutations in the *KRAS* and *BRAF* genes may occur in all patients with GC.

5.1. Limitations and Conclusions

One of the limitations of the current study was the small number of patients with confirmed histological diagnosis of *H. pylori* infection and GC; the authors suggest future studies with larger population sizes in the same field. *H. pylori* can cause molecular changes and genetic mutations in patients with GC and PU. Therefore, it could be stated that *H. pylori* is a carcinogenic agent in GC. The frequency of mutation was higher in *H. pylori*-infected patients with GC compared to their counterparts with PU. DNA sequencing showed *KRAS* and *BRAF* mutations in *H. pylori*-infected GC tissue samples, while no mutations were observed in *H. pylori*-infected PU tissue samples. The considerable prevalence of these mutations in *H. pylori*-infected patients with GC indicated the critical role of these genes in such patients.

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Footnotes

Authors' Contribution: Khatoon Heidari, Hami Kaboosi, and Ailar Jamali: the study design; Khatoon Heidari: the study conduction and collection of samples; Khatoon Heidari, Hami Kaboosi, Ezzat Allah Ghaemi, and Fatemeh Peyravii Ghadikolaii: performing the experiments and analyzing the data. All authors contributed to writing the manuscript. The authors read and approved the final copy of the manuscript.

Conflict of Interests: The authors declared no conflict of interest.

Ethical Approval: The present study protocol was approved by the Ethics Committee of Ayatollah

Amoli Branch, Islamic Azad University (ethical code: IR.IAU.AMOL.REC.23930507971001) and performed in accordance with the Declaration of Helsinki.

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Patient Consent: The participants signed the informed consent forms.

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