

Evaluation of *Helicobacter Pylori* and *NapA* Gene in Paraffin Blocks of Gastric Adenocarcinoma Tissues from Pathology Bank of Shahid Beheshti Hospital, Qom, Iran (2011-2017)

Qom, İran'daki Shahid Beheshti Hastanesi Patoloji Bankası'nda 2011-2017 Yılları Arasındaki Mide Adenokarsinom Dokularının Parafin Bloklarında *Helicobacter Pylori* ve *NapA* Geninin Değerlendirilmesi,

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ABSTRACT

Objective: *Helicobacter pylori* is a known cause of gastrointestinal diseases such as gastritis, gastric ulcers and in severe cases of gastric cancer. Neutrophil-activating protein (NAP) is one of the important markers in bacterial pathogenesis. The aim of this study was to evaluate the presence of *Helicobacter pylori* and *napA* gene in specimens with gastric cancer.

Methods: This study was performed on 67 gastrectomy specimens stored in the pathology ward of Shahid Beheshti Hospital in Qom. The presence of *Helicobacter pylori* infection in cancerous tissues was evaluated by Giemsa staining. After recording the microscopic data, DNA was extracted from paraffin blocks using a commercial kit. Finally, PCR of 16s rRNA and *napA* genes were evaluated.

Results: According to pathological evaluation results, forty gastrectomy cases (59.7%) were diagnosed as gastric adenocarcinoma. The mean age \pm standard deviation (SD) for the patients was 49.12 \pm 18.54 years and was more prevalent in men. The microscopic examination of the tissues showed that all adenocarcinoma samples were also positive for *Helicobacter pylori* (100%-40/40). Intestinal type adenocarcinoma (85%), N1 lymph node metastasis (52.5%), G1 grade (50%), and T2 stage (65%) were the most frequent pathological findings. The results of 16s rRNA gene analysis were in accordance with pathological results and all samples were positive by PCR method as well. All *Helicobacter pylori*-positive samples had no *napA* pathogenic gene.

Conclusion: The results showed that cases of gastric adenocarcinoma in Qom were significant and positive cases of *Helicobacter pylori* infection were high among these samples. In this study, no case of *napA* positive gene was found. It seems that this gene alone is not an independent factor in tumor development and may be influenced by other virulence factors. Therefore, further research is needed to clarify the issue.

Key Words: *Helicobacter pylori*, *napA*, gastric adenocarcinoma

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ÖZET

Amaç: *Helicobacter pylori*, gastrit, mide ülseri gibi gastrointestinal hastalıkların ve ağır mide kanseri vakalarının bilinen bir nedenidir. Nötrofil aktive edici protein (NAP), bakteriyel patogeneizde önemli belirteçlerden biridir. Bu çalışmanın amacı mide kanserli örneklerde *Helicobacter pylori* ve *napA* geninin varlığını değerlendirmektir.

Yöntem: Bu çalışma, Qom'daki Shahid Beheshti Hastanesi patoloji koğuşunda saklanan 67 gastrektomi örneği üzerinde gerçekleştirildi. Kanserli dokularda *Helicobacter pylori* enfeksiyonunun varlığı Giemsa boyama ile değerlendirildi. Mikroskopik verilerin kaydedilmesinden sonra, ticari bir kit kullanılarak parafin bloklarından DNA ekstrakte edildi. Son olarak, 16s rRNA ve *napA* genlerinin PCR'si değerlendirildi.

Bulgular: Patolojik değerlendirme sonuçlarına göre kırk gastrektomi olgusu (% 59.7) mide adenokarsinomu tanısı aldı. Hastaların ortalama yaş \pm standart sapma (SS) 49.12 \pm 18.54 yılı ve erkeklerde daha sıklıkla. Dokuların mikroskopik incelemesi, tüm adenokarsinom örneklerinin *Helicobacter pylori* (% 100 - 40/40) için de pozitif olduğunu gösterdi. Bağırsak tipi adenokarsinom (% 85), N1 lenf nodu metastazı (% 52.5), G1 derecesi (% 50) ve T2 evresi (% 65) en sık görülen patolojik bulguları. 16s rRNA gen analizi sonuçları patolojik sonuçlarla uyumluydu ve tüm numuneler PCR yöntemi ile de pozitif. Tüm *Helicobacter pylori*-pozitif numunelerde *napA* patojenik geni yoktu.

Sonuç: Sonuçlar, Qom'da mide adenokarsinomu vakalarının önemli olduğunu ve bu örnekler arasında pozitif *Helicobacter pylori* enfeksiyonu vakalarının yüksek olduğunu gösterdi. Bu çalışmada *napA* pozitif geni vakasına rastlanmamıştır. Görünüşe göre bu gen tek başına tümör gelişiminde bağımsız bir faktör değildir ve diğer virülans faktörlerinden etkilenebilir. Bu nedenle, konuyu netleştirmek için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Sözcükler: *Helicobacter pylori*, *napA*, mide adenokarsinomu

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INTRODUCTION

Cancer is the main cause of death in both developing and developed countries. According to available statistics, it is predicted that the prevalence of cancer will spread around the world, especially in less developed countries, where about 82% of the world population resides. (1). The complexity of the disease is caused by several factors including environmental and host-associated factors as well as infections (2, 3). Lifestyle has a direct impact on the increased risk of cancer, which may include smoking, poor diet, physical inactivity, and reproductive changes (1).

It has been reported approximately 18.1 million new cases of cancer (17 million excluding non-melanoma skin cancer) and 9.6 million deaths due to cancer (9.5 million excluding non-melanoma skin cancer) all around the world, in 2018. Lung cancer, in both genders, is the most cancer that commonly diagnosed (11.6% of all cases) and the main cause of cancer deaths (18.4% of total cancer deaths), followed by breast cancer in women (11.6%), prostate cancer in men (7.1%) and colorectal cancer (6.1%). Lung cancer is the most common cancer and the main cause of cancer deaths among men, followed by prostate and colorectal cancer (for incidence) and liver and gastric cancer (for mortality). Among women, breast cancer is the most cancer that commonly diagnosed and the main cause of cancer deaths, followed by colorectal and lung cancer for incidence and vice versa (lung and colorectal) for mortality. Cervical cancer ranks fourth for both incidence and mortality (4).

Despite advances in prevention, diagnosis and treatment, gastric cancer is a complex and heterogeneous disease that has raised global health concerns. The disease has still been reported as the main cause of cancer death. It is the fifth most common cancer and the third main cause of death among worldwide malignancies. Fortunately, the incidence and death rate of gastric cancer has dropped dramatically in the past 70 years. However, gastric cancer in Iran is the most common cancer in men and the second most common cancer in women (after breast cancer) and its prevalence has increased in some provinces, especially in northern provinces such as Mazandaran (5, 6).

According to Lauren's classification, gastric cancer is divided into diffuse and intestinal types (7). Diffuse adenocarcinoma is more aggressive and affects young people and does not occur through histological stages; whereas intestinal adenocarcinoma is characterized histologically by developing from the infiltration of inflammatory cells associated with *Helicobacter pylori* to atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately adenocarcinoma (3).

Several pathogenic factors of *Helicobacter pylori* strains have been reported including adhesins, urease, cytotoxin-associated gene A (CagA), Vacuolating cytotoxin A (VacA), neutrophil-activating protein (NAP), etc. that are critical for attachment to epithelial cells and colonization, resulting in the pathogenesis of bacterial infection (8, 9).

Among these factors, *Helicobacter pylori* Neutrophil-activating protein (HP-NAP) is one of the most important pathogenic factors of the bacterium. This protein is able to induce the binding of neutrophils to endothelial cells and to reduce nitroblue tetrazolium (NBT) through the production of reactive oxygen intermediates (ROIs).

Table 1: Sequences of 16s rRNA and *napA* genes primers

Gene	Primers (5'→3')	Size of amplified product(bp)	Reference
16s rRNA	F: CTGGAGAGACTAAGCCCTCC R: ATTACTGACGCTGATTGTGC	110 bp	(11)
<i>napA</i>	F: AATTATGAAGAGTTTGGCGG R: TTCTTTTTCAGCGGTGTAG	223 bp	This study

In a total volume of 25 µl, PCR was run including 10 µl of Master Mix 2X (Ampliqon, Denmark), 1 µl of each primer (Metabion, Germany) (10 pmol/ µl), 3 µl of extracted DNA and 10 µl of sterile distilled water. The Reaction was done at 95 °C for 10 min as initial denaturation, followed by 33 cycles including denaturation (95 °C/ 15 s), Annealing (56 °C/ 30 s for 16s rRNA gene and 52 °C/ 30 s for *napA* gene), extension (72 °C/ 30 s), and final extension (72 °C/10 min) using a Thermocycler (Eppendorf, Germany).

Significant variation in the activity level of the protein has recorded among different strains of *Helicobacter pylori*, indicating a variable level of protein expression (similar to VacA). HP-NAP is contained in the bacterial cytosol and released after autolysis. It can bind to the outer surface of the membrane in a manner similar to the urease enzyme. So, by reaction with carbohydrates, the protein can mediate the attachment of bacteria to the cell surface (10).

Therefore, with the possible role of HP-NAP in the pathogenesis of *H. pylori*, this study was carried out as the first study in the province of Qom to investigate the presence of *Helicobacter pylori* and *napA* pathogenic gene in paraffin-embedded gastric cancer samples in a seven-year period.

METHODS

This study was conducted in paraffin blocks of gastric adenocarcinoma tissues stored in the pathology ward of Shahid Beheshti Hospital, Qom, which was approved by the ethics committee of the university with code IR.IAU.Qom.REC.1397.007.

Pathological evaluation

Using the hospital information system, the records of gastrectomies performed from 2011 till 2017 were investigated and all cases with gastric adenocarcinoma were identified. Then, tissue sections were prepared from the paraffin blocks and after staining by Giemsa, the slides were evaluated by a pathologist. All data were also recorded including demographic information and microscopic findings e.g. type of adenocarcinoma, tumor grade, perineural invasion, lymphovascular invasion, lymph node metastasis, tumor stage, and presence of *Helicobacter pylori* infection.

Primer Design

Two pairs of primers for detecting 16s rRNA and *napA* genes were used in this study. The specific primer for 16s rRNA, as a conserved gene and universal for *H. pylori* diagnosis, was used from the study of Saeidi et al. (11). Using designed primers in this study, the presence of the *napA* gene was also investigated. For this purpose, the sequences deposited at NCBI (<https://www.ncbi.nlm.nih.gov/>) were downloaded and saved by CLC sequence viewer 7.6 software (CLC bio, Aarhus, Denmark). Then, using the alignment of *H. pylori* sequences, suitable sites were identified for primer designing. Gene Runner software was used to evaluate the designed primers for Tm, GC percentage, and secondary structures. Finally, by Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), its attachments to other bacteria were theoretically investigated and confirmed.

DNA extraction and PCR

Paraffin-embedded DNA Extraction Kit (Favorgen, Taiwan) was used to extract genomes from the blocks according to the manufacturer's instructions. The extracted genomes were stored at -20 °C until PCR.

To confirm the presence of *Helicobacter pylori* in the samples that were reported as positive in the pathological evaluation, PCR amplification of the 16s rRNA gene fragment of *H. pylori* was also performed. All positive samples were screened for the presence or absence of *napA* virulence gene using specific primers (Table 1).

Statistical analysis

Age and gender were calculated using mean and standard deviation, and pathologic findings were calculated using frequency distribution by SPSS software version 22.

RESULTS

During 7 years, 67 cases of gastrectomy were recorded in our hospital. By evaluating microscopic slides, 40 (59.7%) cases of gastric adenocarcinoma were diagnosed. The mean age ± SD of patients was 49.12 ± 18.54 years. Among these patients, men accounted for 30 cases (75%) of the population with the highest cancer incidence, compared to women (10 cases, 25%). Tissue evaluation with Giemsa staining showed that all cancer samples were also positive for *Helicobacter pylori* (40/40, 100%) (Fig.1). Intestinal-type adenocarcinoma was the most common (34 cases, 85%). The most common tumor grade identified was G1 (20 cases, 50%), followed by G2 (16 cases, 40%) and G3 (4 cases, 10%). N1 lymphatic metastasis (21 cases, 52.5%) and T2 stage (26 cases, 65%) were also the most important pathologic findings. More information is presented in Table 2.

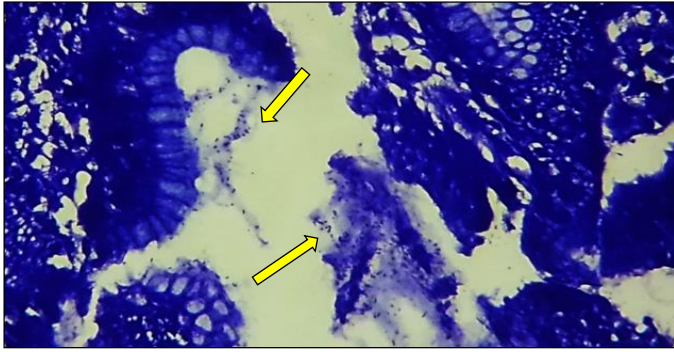


Figure 1: Microscopic image of Giemsa-stained section (magnification x 400) of cancer tissue (arrow: *Helicobacter pylori*).

Table 2: Pathologic findings of the group with cancer. * (NX: regional lymph node cannot be assessed)

Pathologic findings	No.	%	
Adenocarcinoma	Intestinal	34	85
	Diffuse	6	15
Tumor grade	G1	20	50
	G2	16	40
	G3	4	10
Perineural invasion	23	57.5	
Lymphovascular invasion	13	32.5	
Lymph node metastasis	N0	11	27.5
	N1	21	52.5
	N2	5	12.5
	NX*	3	7.5
Tumor stage	T1 (T1a & T1b)	9	22.5
	T2	26	65
	T3	5	12.5

For confirmation of the presence of *Helicobacter pylori*, the 16s rRNA gene was evaluated for all cancerous tissue samples. The PCR results were consistent with the pathological findings and all *H. pylori*-positive paraffin-embedded specimens were also positive by PCR (Fig. 2). The *napA* gene was evaluated on 40 16s rRNA-positive samples and all of them were negative for this gene (0%) (Fig. 3).

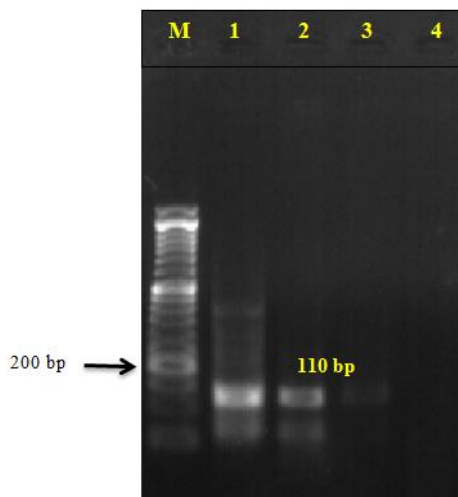


Figure 2: Electrophoresis of 16s rRNA PCR on 1% agarose gel. M: 50bp marker, No. 1-3: *Helicobacter pylori*-positive samples, No. 4: negative control.

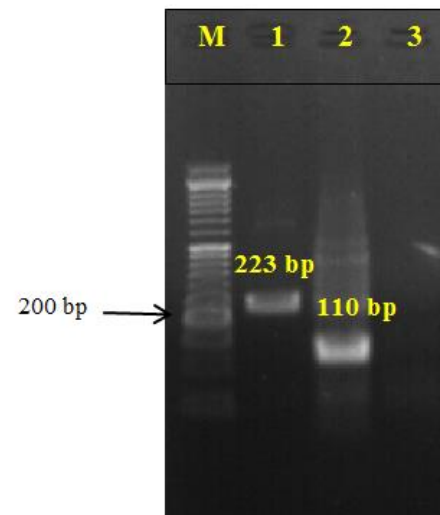


Figure 3: Electrophoresis of *napA* PCR on 1% agarose gel. M: marker 50bp, No. 1: positive control of *napA*, No. 2: positive sample of 16s rRNA gene, No. 3: negative control.

DISCUSSION

Gastric cancer has been known as one of the major causes of death worldwide. More than 90% of gastric cancers are adenocarcinoma and the rest is lymphoma or sarcoma of gastrointestinal stromal tissue. Approximately two-thirds of gastric cancer cases occur in developing countries and 42% in China alone. However, gastric cancer cannot easily be classified as associated with less developed economies.

Statistics showed that the distribution of gastric cancer does not follow a specific geographical pattern. Thus, although some of the most at-risk populations exist in Asian countries such as Japan, Korea, and China, some countries are relatively low in these areas (such as India) (12, 13).

Helicobacter pylori is one of the risk factors for gastric cancer, which is classified as a class 1 carcinogen by the World Health Organization (WHO) (14, 15). *H. pylori* strains have infected approximately >50% of the world's population. There are variations in the prevalence of *H. pylori* infection worldwide and the incidence varies according to age, socioeconomic status, education level, living environment, occupation, and geographical area. Some reports showed that prevalence is highest in Africa, Latin America, the Caribbean, and Asia, especially in developing countries (16, 17).

The clinical symptoms of infection with this bacterium can be classified into four categories: (1) chronic gastritis, which occurs in almost all patients and most are asymptomatic; (2) duodenal ulcer (DU) phenotype, which occurs in 10-15% of infected individuals; (3) the gastric ulcer/adenocarcinoma phenotype, which in 1-3% of infected individuals develops into gastric cancer; and (4) gastric mucosa-associated lymphoma (MALToma), which occurs in 0.1% of infected people (18).

In the current study and based on pathological findings, 59.7% of gastrectomy cases had gastric adenocarcinoma. Most cases of adenocarcinoma were found in men with a prevalence of 75%. Higher rates of gastric adenocarcinoma in men are consistent with other studies. In an epidemiological study of gastric cancer, Crew et al. stated that men are five times more likely than women to have gastric cancer (19). According to a study by Jenabi et al., the prevalence of gastric cancer in Qom and in men (15.9%) was about three times higher than in women (5.7%) (20). In another study by Farhadifar et al. in Ramsar, Iran, out of 144 patients with gastric cancer, 71.5% were male and the rest were female (21).

In this study, 100% of the samples with gastric adenocarcinoma were positive for *Helicobacter pylori*. According to published articles, the prevalence of this bacterium is very high in Iran and is significant in some areas such as Ardabil province (22). According to Yamagata et al. study (23), it appears that the infection to be one of the reasons for the high prevalence of gastric adenocarcinoma in this province, compared to other regions of Iran (one-third of cases). In the report of Maddah and coworker, the prevalence of *Helicobacter pylori* in gastric adenocarcinoma was 92% (24).

The highest number of cancer types was intestinal adenocarcinoma (85%). This type of cancer is reported to be prevalent in high-risk geographic regions such as East Asia, Eastern Europe, Central America and South America, and accounts for the largest international variation of gastric cancer. Therefore, the type of diffuse adenocarcinoma has a more uniform geographical distribution than the former type (25).

Histologic tumor grade divides into 3 categories including well differentiated low-grade (G1), intermediate-grade moderately differentiated (G2), and high-grade poorly differentiated, undifferentiated (G3) adenocarcinoma. According to our results, the G1 with a 50% frequency was the most common tumor grade. Unlike this study, Ahmadi Hedayati et al. in Kurdistan, Iran, reported that the frequency of G2 tumor grade was the highest prevalence (22 cases, 44%) amongst 50 gastric adenocarcinoma patients (26).

The identification of the prognostic factors is important for predicting the survival of patients with gastric cancer and determining treatment options. It is generally acknowledged that the most important factors affecting the prognosis of patients with gastric cancer are the depth of invasion (T stage), lymph node metastasis status (N stage), and perineural invasion (27, 28).

According to the National Comprehensive Cancer Network (NCCN) guidelines, the tumor stage is classified into four categories including T1, T2, T3, and T4. Patients with T1 have a relatively good prognosis. Patients with T3 or T4 have poor prognosis whereas others with T2 show the intermediate stage between primary and advanced gastric cancer. Thus, there is a great deal of variance in the prognosis of patients with T2 gastric cancer (29, 30). T2, with a rate of 65% (26 cases), was the most common tumor stage. Contrary to this result, T3 was the most tumor stage (43.33%) in the report of Keyhanian et al. (31).

Based on the College of American Pathologists (<https://www.cap.org/>), N represents the number of metastatic lymph nodes e.g. N1 indicates 1 to 2 positive nodes. N1 lymph node metastasis (52.5%) showed the highest rate among our patients when compared with N0, N2, and NX. In the study of Zhuo et al., 307 patients had lymph node metastasis that 86 of them were N1 stage (32).

Perineural invasion presents a variety of malignant tumors and can be a sign of tumor metastasis and invasion and shows poor prognosis (33). Among our patients, the perineural invasion was identified with a prevalence of 57.5%. This finding is in agreement with Selçukbiricik et al., which reported the presence of perineural invasion in 211 of 287 gastric cancers (73%) (34).

Molecular assay of the 16s rRNA gene showed that all positive samples in the pathological evaluations were also positive for *H. pylori*. This result indicated that both methods were the same in sensitivity and can be used to diagnose infection. In a study by Lu et al., a comparison of five molecular PCR methods with different genes including urease A (*ureA*), 16s rRNA, random chromosome sequence, 26-kDa species-specific antigen (SSA), and urease C (*ureC*) for the detection of *Helicobacter pylori* in gastric tissues were evaluated. They showed that the 16s rRNA, SSA, and *ureC* genes detected in 100% of culture-positive specimens (24/24 samples). Thus, these genes detected can be utilized for the diagnosis of bacteria in gastric biopsy (35).

Bacterial pathogenic factors play an important role in the progression of gastritis to gastric cancer; therefore their detection can help to identify the strains in the different regions. Miehke et al. (2011) showed that infection with positive strains for *cagA+* and *vacAs1/m1* were more associated with the progression of gastric cancer (36).

Koehler et al. (2003) evaluated *Helicobacter pylori* genomes isolated from 92 paraffin-embedded tissue specimens of patients with gastric adenocarcinoma and MALT lymphoma. They found that the prevalence of *iceA1* and *iceA2* genes was 6.5 times higher in patients with adenocarcinoma (37).

In this study, the *napA* gene was selected and evaluated. Although some studies have suggested a link between this gene and cancer, none of the samples were positive for the gene. Long et al. (2009) showed that serum positive for HP-NAP specific antibodies in gastric cancer patients was significantly higher than in chronic gastritis and healthy (control) groups and HP-NAP may contribute to the development of gastric cancer by producing interleukin-8 in gastric epithelial cells (38).

Contrary to the done study by Long et al., another report showed that serum level of anti-NapA antibody was associated with reduced risk of gastric cancer and that this protein could not be used as an independent indicator in screening and diagnosis of gastric cancer (39).

Surprisingly, Shakeri et al. also reported that NapA was significantly associated with a reduced risk of gastric cardia and noncardia adenocarcinomas (40). Therefore, these findings may justify the absence of this gene in our study strains.

CONCLUSION

Due to the presence of *Helicobacter pylori* in all adenocarcinoma samples; there may be a link between the existence of bacteria and cancer. The *napA* gene was not found in the samples and appears to have played no significant role in the carcinogenicity of the bacteria. It seems that *napA* alone is not a virulence factor in the progression of gastritis to gastric cancer and may be influenced by other virulence-associated genes of the bacteria. Therefore, the evaluation of other genes is recommended.

Conflict of interest

No conflict of interest was declared by the authors.

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