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Association of D299G Polymorphism of TLR4 Gene and CagA Virulence Factor of *H. pylori* among the Iranian Patients with Colorectal Cancer

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Abstract

Background: Colorectal cancer (CRC) represents 9% of all malignancies globally. TLR4 gene defenses against *Helicobacter pylori* infection (HPI), so its mutations are a risk factor for CRC. As there is a correlation between (HPI) and gastric cancer, we investigated whether there is an association between CagA virulence factor in HPI and D299G polymorphism of TLR4 gene with developing CRC among Iranians.

Methods: This retrospective study included 85 biopsies of confirmed colorectal lesions out of 230 subjects, which were divided into two age groups. Single nucleotide polymorphism (SNP) D299G in the TLR4 gene was assessed using Tetra-primer ARMS-PCR. The expression of TLR4 and the CagA virulence factor in *H.pylori* was assessed using real-time PCR (RT-PCR).

Results: Chi-squared test showed genotype frequencies of GG were 79% and 62%in patients 51> and 51<years, respectively. Logistic regression showed a positive association between the presence of CagA and a high GG allele (p=0.002). The odd ratio was predicted as 4.80 using the Hardy-Weinberg equilibrium assumption. Iranians with CagA and high GG of D299G were four times more likely to develop CRC than their peers with AA allele.

Conclusion: *H. pylori*-positive CagA has a higher ability to escape from the immune response. D299G polymorphism of TLR4 gene full of GG allele is an influential risk factor in developing CRC. Hence finding *H. pylori*-positive CagA should be noticed as a marker of the TLR4 gene full of GG allele in screening plans.

Keywords: *H.pylori*, Virulence Factor CagA, Toll-like Receptor4, Colorectal Cancer

Conflicts of Interest: *None declared*
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Introduction

Colorectal cancer (CRC) represents 9% of all malignancies globally, the second leading cause of death in the United States. The ubiquity of CRC has an increasing pattern in the United States, Canada, and Australia. Environmental and genetic factors are the most risk factor for developing CRC (1-3). In addition, innate and adaptive immune systems contribute to coping with infectious diseases. Communication between these immune systems is essential for eliminating infections. Toll-like receptors (TLRs) are single-pass membrane proteins that belong to pattern recognition receptors (PRRs). They are expressed on the surface of innate immune cells such as macrophag-

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↑What is "already known" in this topic:
The evidence about its correlation with virulence factor CagA carrying *H.pylori* and TLR4 gene polymorphism (D299G) has remained controversial and conflicting in diverse areas.

→What this article adds:
Iranian patients with *H. pylori*, and AA genotype of TLR4, are less likely to develop CRC than others with GG. Receiving a timely diagnosis and early treatment demands choosing the best screening test ends up meeting an acceptable level of expense and accuracy.

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Table 1. Primer design for Tetra-ARMS PCR

SNP	Sequencing Primers	Product Size
Asp299Gly(A>G)	F Outer: 5'_TGA ACC CTA TGA ACT TTA TCC-3' R Outer: 5'_GTT AAC TAA TTC TAA ATG TTG CCATC-3' F Inner: 5'_GCA TAC TTA GAC TAC CTC GAA GA-3' R Inner: 5'_CAA ACA ATT AAA TAA GTC AAT AC-3'	Outers: 385 A Allele: 147 G Allele: 292

es and dendritic cells. These receptors bind to conserved structural molecules on microbes. So far, ten types of TLRs have been identified in humans. Amongst them, overexpression of TLR4 and TLR2 are correlated with CRC. Single nucleotide polymorphisms (SNPs) in TLR genes, also known as D299G SNP, have been claimed to have a pivotal marker for predicting numerous malignancies including CRC (4).

SNOs are the common mutations in TLR4 promotors, while TLR4 is responsible for detecting lipopolysaccharide (LPS) on the surface of gram-negative bacteria like *Helicobacter pylori* (*H. pylori*). Not surprisingly, this loss-of-function mutation reduces TLR4 responsiveness to LPS. Therefore, it is related to several diseases like atherosclerosis, asthma, and H.pylori infection-induced gastric cancer (4, 5). Recently, a study carried out among the Caucasian population has demonstrated the role of D299G in Crohn's disease and ulcerative colitis (6). Another study was done amongst the Tunisian population that demonstrated a relation between TLR4 D299G polymorphism and CRC significantly associated with future clinical variables (7) .Similarly, it was reported that the incidence of CRC rose by three times in the European population with D299G polymorphisms of the TLR4 gene (8-10).

H. pylori are one gram-negative bacteria infecting half of the global population. Studies have provided evidence that *H. pylori* carry several specific virulence factors, divided into colonization, immune escape, and disease induction categories, which influence its pathogenicity. Cytotoxin-associated gene A (CagA) belongs to the disease induction and immune escape category (11, 12). It is proven that the presence of CagA in *H. pylori* is associated with gastric cancer and peptic ulcer disease. Probably its immune escape ability hinders hosts' innate immune system responses to inflammation. Consequently, pro-inflammatory cytokines activate the underlying cell-signaling cascade, mediated by TLRs (13, 14). In most cases, the level of inflammatory responses is directly linked to *H. pylori* pathogenesis. Several studies have shown a positive correlation between *H. pylori* infection (HPI) and gastric cancer. Although there is no solid evidence to confirm the correlation between HPI and CRC, numerous studies have noticed conflicting results (8, 9, 15). Hence, we investigate the correlation between the presence of CagA and TLR4 gene polymorphism (D299G) in provided CRC samples. TLR4 gene SNP rs4986790 was detected by Tetra-ARMS PCR, CagA and TLR4 expression were measured using real-time PCR. Genotype frequencies were calculated, and statistical analyses were performed using the chi-squared test.

Methods

Sample Recruitment

In this retrospective study, first, written informed consent to use samples was obtained from all participants. The entire experimental steps were performed after receiving permission from the National Ethics Committee in Biomedical Research (Medical Research Ethics Code: IR.IUMS.REC.1399.515). Next, 230 biopsies of colorectal lesions were collected from patients referred to the pathology department of Imam Khomeini hospital, Karaj, Iran. Then Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples were stained with Hematoxylin and Eosin (H&E).in the end, 85 CRC samples, confirmed by a pathologist, were collected for further investigation.

DNA preparation

DNA was isolated after proteinase K digestion using a DNA extraction kit (Cinnagene, DN8115C), according to the manufacturer's instructions

Then isolated DNA was purified and qualified via nanodrop spectrometry (Thermo Fisher Scientific, USA).

Tetra-ARMS PCR

Tetra-primer ARMS-PCR was applied to assess The SNP of D299G in the TLR4 gene. Four primers were designed using Oligo primer, including two Inner and two Outer primers. PCR was carried out based on the manufacturer's recommended procedures. Primer design and restriction enzymes are represented in (Table 1).

Real-time PCR

RNA was isolated using the Trizol method. Then the isolated RNA visualized using 1% agarose gel electrophoresis was quantified using Nanodrop (Thermo Scientific) spectrophotometer and was stored at 80°C.The cDNA was synthesized using QuantiTect® Reverse Transcription kit (QIAGEN) and visualized by gel electrophoresis. Next, real-time PCR was performed by ABI 7500 RT-PCR system; primers are shown in Table 2 as well. The denaturation program was 94 °C for 11 minutes, followed by annealing (61°C for 1 minute) and extension (72 °C for 11 minutes). GADPH was used as an internal control.

Statistical Analysis

Genotype frequencies were analyzed by the chi-squared test using Med Calc ver. 12.1.4 Software. Logistic regres-

Table 2. Primer design for RT-PCR

Gene		Sequence (5'→3')
TLR4	Forward	AATCTAGAGCACTTGGACCTTTCC
	Reverse	GGGTTCAGGGACAGGTCTAAAGA
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAAGATGGTGATGGGATTTC

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sion was done with the Hardy-Weinberg equilibrium assumption to predict the odds ratio. A P-value less than 0.05 were considered statistically significant.

Results

In brief, 85 confirmed CRC lesions were selected out of 230 subjects. Demographic results showed that more than a quarter of patients aged 41-50. The samples were then divided into two age groups: 51< and 51≥ years old .They were nearly equally distributed for age in 2 groups, 51% were older than 51 vs. 49% were younger than 51. Our results showed that about half (51 %) of patients were older than 51-year-old. Since the G allele is considered +as a risk factor, the patients with AG and GG *genotypes were*more susceptible to developing CRC than the AA genotype.

The expression level of TLR4 and genotype frequencies

Relative TLR4 mRNA expression level was measured using RT-PCR. After total RNA extraction, cDNA was amplified and assessed by the gel electrophoresis method. We also compared the expression level of TLR4 in both age groups. There was a two-fold decrease in the expression of the TLR4 gene in subjects younger 51-year-old (p=0.05). The expression level of GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was measured as a common housekeeping gene to normalize data (Fig. 1).

The frequency of D299G in the TLR4 gene, including GG, AG, and AA, was analyzed using tetra-ARMS PCR. As can be seen in Table 3 in patients older than 51 years, the percentage of GG, AG and AA genotype frequencies were 79%, 16%, 5%, respectively, and it was 62%, 21%, 17%, respectively, in case of patients younger than 51 (Fig. 2**)**. Considering the G allele as a risk factor, the AG and GG genotype patients are more likely to develop CRC than the AA genotype (Table 3).

Lymph node metastases

Since lymph node metastases are accepted as a significant prognostic factor in CRC, we also assessed them, their metastases, and their correlation with gender. A vast majority of metastasis (76%) was detected in women with a 0.78 linear correlation coefficient, which indicates a positive relationship between feminine sexuality and lymph node metastases (P<0.05), whereas we did not find a significant correlation between tumor size and gender (Fig. 3).

H. pylori cagA *positive detection and the correlations*

Our result showed that among CRC confirmed patients younger than 51 y,33 out of 42 presented the virulence factor CagA, while the older age group was 28 out of 43. There was no significant difference between the 2 age groups in presenting *cagA virulence*factor (Fig. 4, Table 4). In addition, the Chi-Square test was applied to find the correlation between presenting the virulence factor CagA in H. pylori and carrying different genotypes of D299G in the TLR4 among the CRC samples. Our result showed a positive relation between H. *pylori virulence factor cagA*

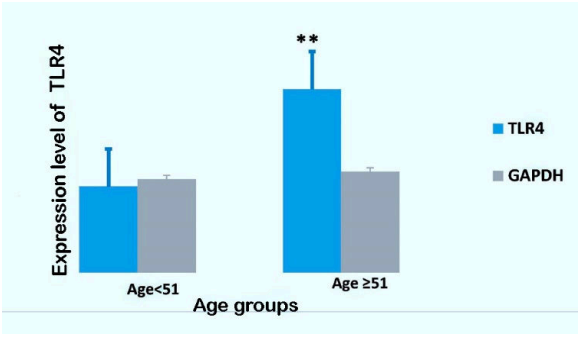

Fig. 1. Different TLR4 expression levels in two-age-group. GAPDH expression profiles were used to normalize gene expression data (p<0.05) (n=85).

Table 3. The percentage of genotype frequencies of D299G polymorphism in two age groups

Allele	Age < 51 and	Age ≥ 51	*P*
GG	62%	79%	
AG	21%	16%	0.004
AA	17%	5%	

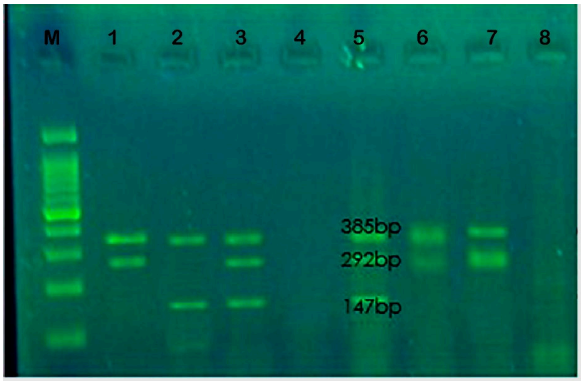

Fig. 2. Agarose gel electrophoresis after Tetra-ARMS PCR. Lane 1, 6, and 7 show GG genotypes; lane 2 and lane 3 show AA and AG genotype, respectively. Lane 4 and 8 show negative control, and M shows the ladder (n=85).

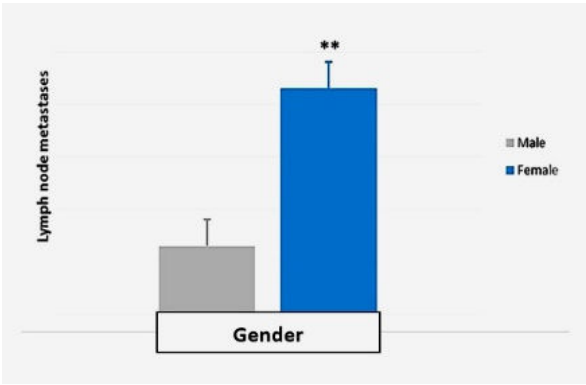

Fig. 3. Gender differences of lymph node metastases in colorectal cancer. Women are more likely to have lymph node metastases compared to men (p<0.05) (n=85).

and the GG genotype of D299G. (p=0.002, odd ratio=4.80, Table 5). Because CagA influences the bacteria's immune escape and disease induction abilities, our result could be interpreted as follows: *H. pylori*-infected subjects with GG genotype are four times more likely to develop CRC than their peers with AA genotype. Therefore, our results suggested that the association between D299G

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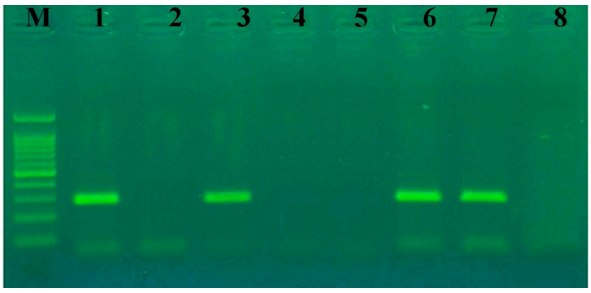

Fig. 4. Agarose gel electrophoresis for CagA. Lane M shows the ladder, Lane1 shows positive control; Lane3, 6, and 7 show H.pilory positive samples; Lane2, 4, and 5 show H.pilory negative samples; Lane8 shows negative control (n=85).

Table 4. The frequency of H. pylori virulence factor A CagA between two age groups (n=85)

Age groups	CagA Positive	CagA Negative	*P*
Age <51 and	33	9	0.168
Age≥51	28	14	
Total	61	15	

Table 5. The relation between CagA positive H. pylori and genotype frequencies of D299G polymorphism in TLR4 gene in two different age groups.

Allele	CagA+ age <51 (n=33)	CagA+ age≥51 (n=28)	Odd Ratio	*P*
GG	64%	82%	1	
AG	21%	14%	1.92	0.002
AA	15%	4%	4.80	

polymorphism and *H. pylori* infection should be noted when designing a CRC screening program for the Iranian population.

Discussion

We investigated the association between D299G polymorphism in the TLR4 gene and *H. pylori* infections as the two major causes of developing CRC The relation between TLRs receptors and the pathophysiology of CRC is not fully understood. In contrast, several studies suggested that TLRs, associated with intracellular pathways, could increase the risk of inflammatory bowel diseases and CRC (16-18). Accordingly, Pimentel-Nunes *et al.* showed that either TLRs overexpression or TLR inhibitors under expression could play a significant role in carcinogenesis and tumor progression (19). TLR4 is one of the TLRs families consisting of 839 amino acids located on the chromosome. This functionally active receptor expresses in human colon cancer cells as well, where it helps cancerous cells escape from the immune response by downregulating the expression level of resistance to immunosuppressive factors, resulting in a poor prognosis (20-22). Wang and colleagues reported that TLR4/MYD88 signaling pathways contributed to CRC tumorigenesis, sporadic CRC, and colitis-associated cancers (23). Another study by O'Leary *et al*. reported that NF-κB activation through the MYD88 pathway enhances the expression of pro-inflammatory cytokines (24). Inconsistency, various *in vitro* and *in vivo* studies reported that mice with TLR4 deficiency show protection against colon carcinogenesis. Single nucleotide polymorphism (SNP) in exon 4 of the human TLR4 gene caused a missense mutation called D299G polymorphism (25). Although this mutation does not affect the dimerization of TLR4, it alters the conformational properties of the D299G site that further affects ligand binding and related signaling pathways (26, 27). The D299G variants show neoplastic progression in Caco-2 intestinal cells correlated with CRC (28) .Messaritakis *et al*. also reported a significant correlation between two polymorphisms in the TLR4 gene (Asp299Gly and Thr399Ile) and early CRC in 397 patients. Their findings confirmed that these polymorphisms are significantly related to CRC's poor prognosis (29). However, Davoodi and Seow demonstrated that D299G and Thr399Ile polymorphisms are not associated with CRC risk (30).

Furthermore, Semlali *et al.* investigated the correlation between four SNPs of the TLR4 gene in CRC in Saudi Arabia. They showed a positive correlation between D299G prostate and gastric cancers but not related to colon cancer (31). However, our results show a positive correlation between D299G polymorphism in the CRC among the Iranians. Furthermore, gene frequency analysis showed that the G allele is the susceptibility factor, which means people with AG and GG genotypes are more likely to develop CRC. There is solid evidence that subjects with H. pylori-infected populations are more likely to develop gastric carcinoma (32).

It should be noted that the pathogenicity of *H. pylori* highly depends on which specific virulence factors are presented. These virulence factors are divided into three categories according to their effects, including colonization, immune escape, and disease induction. CagA that has been detected in this study belongs to both immune escape and disease induction categories(15, 33). The presence of CagA is associated with gastric cancer and peptic ulcer disease, which could justify why there are various controversial reports about the relationship between *H. pylori* and CRC incidence. Chen *et al*. showed the likelihood of a relation between *H. pylori* infection and developing colorectal adenomatous polyps (34).

On the one hand, many studies reported a correlation between *H. pylori* infection and CRC, with the odds ratio ranging from 1.15 to 10 (34-37). On the other hand, some studies failed to prove a cause-effect relationship between H.pylori infection and the developing CRC in the United States (33, 38, 39). It is confirmed that H.pylori batteries which express CagA, release inflammatory cytokines such as IL-8. These inflammatory cytokines are related to increasing the production and secretion of gastrin, which can lead to chronic atrophic gastritis. Moreover, Shmuely *et al.* reported a relation between CagA-positive H.pylori with colorectal cancer (40). Selgrad *et al.* showed that the expression of the CagA gene resulting from Pylori infection increases the risk of clonic neoplasms (41)

Our result confirmed that in patients with CRC, there is a positive relation between *H. pylori* virulence factor CagA and the GG genotype of D299G (p=0.002, odd ratio=4.80, Table 5). Because CagA influences the bacteria's immune escape and disease induction abilities, and the G

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allele is known as the Risk factor, it could be concluded that H. pylori-infected individuals with GG genotype are four times more likely to develop CRC than a peer with AA genotype. Therefore, our results suggested that the relation between D299G polymorphism and the *H. pylori* with CagA virulence factor should be considered in the CRC screening programs designed for the Iranian population. *H. pylori*-infected carrying CagA subjects with GG genotype are four times more likely to develop CRC than a peer with an AA genotype.

Conclusion

The Probability of developing CRC in Iranian patients with CagA carrying *H. pylori* with GG genotype of D299G polymorphism is four times higher than the peers with AA genotype. To sum up, we suggested that when designing a CRC screening program for the Iranian population, the presence of *H. pylori* carrying CagA and D299G polymorphism full of GG allele should be noticed so that high-risk individuals could possibly receive timely diagnosis and treatment.

Acknowledgment

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Ethical considerations

The study were performed after receiving the required ethical approval and permission from the National Ethics Committee in Biomedical Research (Medical Research Ethics Code: IR.IUMS.REC.1399.515), provided by the Ethical Committee of Iran University of Medical Sciences.

Conflict of Interests

The authors declare that they have no competing interests.

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