The Relation Between Host TLR9 -1486T/C, rs187084 Gene Polymorphisms and *H. Pylori cagA, sodB, hsp60 and vacA* Virulence Genes Among Gastric Cancer Patients

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Research Article

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Abstract

Purpose: To identify different genotypes of TLR9 -1486T/C (rs187084) in patients with gastric cancer and reveal their relation to *H. pylori* virulence genes (*cagA, sodB, hsp60 and vacA)*.

Methods: Patients with gastric cancer were recruited to our study, diagnosed both endoscopically and histo-pathologically. *H. pylori* strains were isolated from gastric samples by culture and PCR amplification of *glmM* gene. Virulence genes of *H. pylori*; *cagA, sodB, Hsp60 and vacA,* were detected by multiplex PCR. Blood samples were used for genotyping of TLR9 -1486T/C (rs187084) by PCR-RFLP*.*

***Results*:** Out of 132 patients with gastric cancer, 106 (80.3%) were positive for *H. pylori* by both culture and PCR. Similar number of healthy participants were recruited as controls. The prevalence of virulence genes among the isolated *H. pylori* were 90.6%, 70.8%, 83.0% *and* 95.3% for *cagA, sodB, hsp60 and vacA* respectively. The *vacA* gene alleles had a prevalence of 95.3% for *vacAs1/s2*, 52.8% for *vacAm1* and 42.5% for *vacAm2*. The CC genotype of TLR9 -1486T/C had a significantly higher frequency in gastric cancer patients when compared to healthy participants (P = 0.045). Furthermore, the CC genotype demonstrated a significantly higher association with *H. pylori* strains carrying *sodB*, *hsp60* and *vacAm1* virulence genes (P = 0.021, P = 0.049 and P = 0.048 respectively).

***Conclusions*:** Patients with CC genotype of TLR9 -1486T/C (rs187084) are at higher risk for development of gastric cancer and its co-existence with *H. pylori* strains carrying *sodB*, *hsp60* or *vacAm1* virulence genes might have a synergistic effect in development of gastric cancer.

Introduction

Helicobacter pylori (*H. pylori*) described as spiral-shaped Gram-negative bacteria that can infect gastric mucosa in more than half of the population all over the world (1). Such infection disturbs the homeostasis of the gastric mucosa and induces the release of inflammatory cytokines (2). Subsequently high association was described between *H. pylori* infection and several gastric pathologies as chronic gastritis and gastric cancer (3, 4). Moreover, the WHO has classified *H. pylori* as a type one carcinogen because of its high association with gastric cancer (5). The development of such different gastric diseases has been linked to the interaction between *H. pylori* virulence factors, the host genetics and immune responses as well as environmental factors (6, 7).

The progress of *H. pylori* disease is influenced by alterations in the host immune components including Toll-like receptors (TLRs) through their prominent role in activation of the immune system both innate and adaptive arms following infection (8, 9). These receptors are known as pattern recognition receptors as they identify pathogen associated molecular patterns (PAMPs) present in most pathogens (10, 11). During *H. pylori* infection, TLRs located on immune cells and gastric local epithelium identifies various PAMPs present on that pathogen.

TLR9 detects unmethylated CpG oligonucleotides present abundantly in bacterial DNA (12). As TLR9 is located inside the intracellular endosomes, its activation requires intracellular transfer of unmethylated CpG oligonucleotides through endocytosis (13). They are expressed by gastric epithelial cells and contribute considerably to immune recognition and signaling following *H. pylori* infection. Moreover, the proper TLRs activation is vital for gut protection and recovery from injury (14).

Dys-regulation of TLRs signaling may result in unbalanced production of inflammatory cytokines with subsequent chronic inflammation which promotes the development of gastric cancer (14). Therefore, single nucleotide polymorphism of TLRs genes such as TLR9-1486 TC (rs187084), may modulate the disease pathogenesis and affect the possibility to develop such disease (11, 15).

Different virulence agents of *H. pylori* can be used as tools to determine the clinical outcomes of the infection (3, 16). The cytotoxin-associated gene A (CagA) is considered one of the main toxins of *H. pylori* (17), that can cause morphological alterations in the host cell triggering cell differentiation and multiplication which can help in the development of gastric cancer (18). The vacuolating cytotoxin (VacA) is another effective toxin produced by *H. pylori* strains and encoded by *vacA gene*. It is a pore-inducing toxin that triggers apoptosis through induction of epithelial cellular vacuolation of the stomach (19).

The superoxide dismutase enzyme (SOD), encoded by the *sodB* gene, is an important bacterial enzyme that helps *H. pylori* strains survival (20, 21). Furthermore, heat shock protein 60 (Hsp60) is a protein that is expressed abundantly by *H. pylori* that acts as molecular chaperone which guard unfolded proteins against acid accumulation (1, 22).

The current study was undertaken to identify different genotypes of TLR9 -1486T/C (rs187084) in patients with gastric cancer and reveal their relation to *H. pylori* virulence genes (*cagA, sodB, hsp60 and vacA)*.

Patients And Methods

Patients selection:

This study was conducted over a period of 15 month (from December 2019 to February 2021). We have recruited 132 patients with gastric cancer at the Gastro-Enterology Surgical Center, Mansoura University, Egypt. They have presented initially with gastric symptoms that was confirmed endoscopically and histo-pathologically to be gastric cancer. Healthy participants who had gastric endoscopy procedures and were proved to be free of any gastric pathology and negative for *H. pylori* infection were recruited as a control group. The epidemiological and clinical data of all participants in the present study were recorded.

We have excluded any participant who fulfilled one or more of the set exclusion criteria that included previous gastric surgery, the use of anti-*H. pylori* eradication therapy, antibiotics, anti-inflammatory

agents, proton pump inhibitors, chemotherapeutic drugs or radiotherapy within one month before the endoscopy procedure.

Samples collection:

A total of 238 stomach biopsies (each of size of 5 mm × 5 mm) were collected by clinicians during the performance of diagnostic gastric endoscopy procedures. Obtained biopsies were stored on ice and immediately transferred to Medical Microbiology and Immunology department, Mansoura University, Egypt for further processing.

A peripheral blood sample of 10 ml was collected under complete aseptic precautions from each study participant for investigating TLR9 gene polymorphisms (TLR9 -1486T/C, rs187084 SNPs).

Isolation of H. pylori from gastric tissue samples:

Collected biopsies were inoculated in sterile tubes with brain heart infusion(BHI) broth (Oxoid- UK), then homogenized by a scalpel on a sterile slide. Homogenized samples were cultured on Colombia agar (Oxoid- UK) plates containing 10% of freshly defibrinated sheep's blood. Besides, plates were supplemented with amphotericin B (4 mg/L), vancomycin (10 mg/L), polymyxin B (10 mg/L), and trimethoprim (5 mg/L) antibiotics (Oxoid- UK). Cultured plates were incubated under microaerophilic circumstances (Campy pack systems, BBL,Cockeysville, Maryland, USA) at 37°C for 3 days (23).

Culture plates were examined for colonies after 3 days where *H. pylori* isolates were identified by having small, translucent and round colonies. Further recognition of *H. pylori* isolates was conducted by Gram stained films followed by biochemical reactions (positive catalase, urease, and oxidase). Suspensions of *H. pylori* strains were prepared using BHI broth supplemented with 20% glycerol and then kept at -20°C for further analysis (24).

Molecular confirmation of isolated H. pylori strains through amplification of glmM gene.

Whole genomic DNA was obtained from cultured isolates using (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany), in line with the provider's rules then the resulted DNA was kept −20°C until further completing of laboratory work.

The set of primers used to amplify the targeted gene was mentioned in Table 1 (5). The PCR was carried out in a reaction mixture (25 µL) containing 12.5 µL master mix (Fermentas, Germany), 5 µL DNA, 0.2 µL of each primer (10 pmol) and 7.1 µL nuclease - free water (25).

Table 1
Sequences of sets of primers used

Gene targeted	Sequence	Size of amplified product (bp)	Ref
glmM	F-5'AAGCTTTTAGGGGTGTTAGGGGTTT3' R 5'AAGCTTACTTTCTAACACTAACGC3'	294	5
cagA	F-5'GATAACAGGCAAGCTTTTGAGG3' R-5'CTGCAAAAGATTGTTTGGCAG3'	349	27
sodB	F-5'GCCCTGTGGCGTTTGATTTCC3' R-5'CATGCTCCCACACATCCACC3'	425	21
hsp60	F-5'GCTCCAAGCATCACCAAAGACG3' R-5'GCGGTTTGCCCTCTTTCATGG3',	603	21
vacA	F-5'CAATCGTGTGGGTTCTGGAGC3' R-5'GCCGATATGCAAATGAGCCGC3,	678	21
vacAs1/s2	F: 5' ATGGAAATACAACAAACACAC3' R: 5'CTGCTTGAATGCGCCAAAC3'	259	28
vacAm1	F:5'GGTCAAAATGCGGTCATGG3' R: 5'CCATTGGTACCTGTAGAAAC3'	290	28
vacAm2	F: 5'CATAACTAGCGCCTTGCAC3' R: 5'GGAGCCC*CAG*GAAACATTG3'	352	28
TLR9 -1486T/C, rs187084	5'TTCATTCATTCAGCCTTCACTCA 3', 5' GAGTCAAAGCCACAGTCCACA 3'	490	30

PCR started by an initial denaturation at 94°C for five minutes, then 40 cycles of denaturation for sixty seconds at 94°C, annealing for ninety seconds at 55°C, and extension for 120 seconds at 72°C. The final extension was performed at 72°C for seven minutes (25).

Molecular detection of cagA, sodB, hsp60, and vacA virulence genes of H. pylori using multiplex PCR

Multiplex PCR was undertaken following the coming steps simultaneously: beginning with incubation for 5 minutes at 95°C; then 34 cycles were run, each consisting of one minute at 94°C, then another one minute for primer annealing at 55°C, followed by extension for 60 seconds at 72°C; to be finished with the final extension step at 72°C for ten minutes (26). The reaction volume was 25 µL that contained PCR master mix (Fermentas, Germany), besides 5 µL of DNA, and 0.2 µL of each primer. PCR products were

then electrophoresed (27). Standard strain (ATCC26695) was used as a positive control (21). Primer sets used were supplemented in table (1)

Molecular detection of different vacA gene alleles in vacA positive H. pylori isolates (vacAs1/s2, vacAm1 and vacAm2)

PCR was conducted by applying the following cycling parameters: first; 95°C for 5 minutes, second; 35 cycles [95°C for 30 seconds, 54°C for 30 seconds and 72°C for 16, 18 and 21 seconds respectively according to the required allele to be amplified *(vacAs1/s2*, *vacAm1* and *vacAm2*)]. Then final extension at 72°C for 10 minutes (28). Sets of primers used were listed in table (1).

Genotyping of TLR9 -1486T/C, rs187084 polymorphisms by PCR-RFLP

Genomic DNA was extracted from the obtained buffy coat (leukocyte-enriched fraction of whole blood) by using Gene JET Whole Blood Genomic DNA purification Mini kit (Fermentas Life Sciences, Canada) according to provider's guidelines then exposed to PCR-RFLP. Blood samples were subjected to centrifugation at 2,500 ·g for 10 minutes to release 3 different layers: the upper clear layer containing plasma; the intermediate buffy coat and the bottom layer of concentrated erythrocytes.

PCR reaction was run with thermal cycling conditions of 4 minutes at 95°C then 35 cycles each starting with 30 seconds at 95°C followed by 20 seconds at 60°C, and 30 seconds at 72°C to be ended with final extension for 5 minutes at 72°C to produce a DNA piece of 490 bp (29). The primers used were illustrated in table (1) (30).

Then the DNA products were digested using AflII restriction enzyme (Thermo Scientific, EU, Lithuania) by incubation for three hours at 37°C to yield one of three variants: two fragments of 192 bp, and 327 bp that indicated TT allele, or three fragments of 192 bp, 327 bp, and 490 bp that proved presence of TC allele or an intact PCR fragment of 490 bp indicating presence of CC allele (29).

Statistical analysis

Obtained data were evaluated by the computer program SPSS (Statistical package for social science) version 22.0. Descriptive items were shown as means ± standard deviation (SD) or frequency (number-percent). P-values less than 0.05 were significant.

The relation between genotypes and the risk of gastric cancer was detected by calculating the odd ratios (ORs) and 95% confidence interval (CIs). We applied Hardy–Weinberg equilibrium to compare genotype frequencies we observed to the expected ones in studied healthy control. The relation between virulence genes of *H. pylori* and TLR9-1486T/C, rs187084 genotypes in gastric cancer patients was identified using Chi square test.

Results

This study was enrolled over a period between December 2019 and February 2021, where 132 patients examined at Gastro-Enterology Surgical Center, Mansoura University, Egypt. They were diagnosed to have gastric cancer by gastric endoscopy followed by histo-pathological analysis. We have included 106 subjects who had gastric cancer and were positive for *H. pylori* infection. A similar number of 106 subjects were free of any gastric pathology and negative for *H. pylori* infection were included as a control group. No significant difference was found in mean ±SD between both groups with or without gastric cancer; 56.55±8.63 years versus 53.27±8.55years, (P=0.93) as shown in Table 2.

Table 2
Demographic and histo-pathological data of included study subjects

Variable	Gastric cancer patients	Healthy study participants	P-value
Age (in years)	56.55±8.63	53.27±8.55	0.93
Sex			
Male	63 (59.4)	59 (55.7%)	0.76
Female	43 (40.6%)	47 (44.3%)	
Differentiation of the tumor			
Well differentiated tumor	16 (15.1%)	NA	-
Moderately differentiated tumor	35 (33.0%)		
Poorly differentiated tumor	55 (51.9%)		
Values are given as mean ± SD, or number (percentage).			
NA: Not applicable			

Sex distribution was similar in studied groups; 59.4% of gastric cancer patients were males and 40.6% were females versus 55.7% males and 44.3% females in control group (P=0.76). Gastric cancer lesions were classified according to histo-pathological examination into well differentiated tumors (15.1%), moderately differentiated tumors (33.0%) and poorly differentiated tumors (51.9%) (Table 2).

In the current study, the collected gastric tissue samples were cultured in order to isolate *H. pylori* strains. Colonies of *H. pylori* were identified by Gram-stained smears, biochemical reactions and PCR amplification of *glmM* gene. Of the 132 gastric cancer patients, 106 (80.3%) gave positive results for *H. pylori* by both culture and PCR whereas 26 (19.7%) were negative. The results were statically significant (P value = 0.000) (Table 3).

Table 3
Distribution of *H. pylori* among gastric tissue samples in studied gastric cancer patients

	Number	%	P value
Positive	106	80.3	0.000*
Negative	26	19.7	
Total	132 (100%)		

Multiplex PCR was performed for the detection of virulence genes among the isolated 106 *H. pylori* strains. The conducted PCR revealed that the prevalence of *cagA, sodB, hsp60*, and *vacA* genes were 90.6%, 70.8%, 83.0% and 95.3% respectively. Regarding the *vacA* gene alleles, they had a prevalence of 95.3% for *vacAs1/s2*, 52.8% for *vacAm1* and 42.5% for *vacAm2* (Table 4).

Table 4
Distribution of studied virulence genes in *H. pylori* strains isolated from gastric cancer patients

Virulence gene	Number N=106	%	P-value
cagA			
Positive	96	90.6	0.000*
Negative	10	9.4	
sodB			
Positive	75	70.8	0.000*
Negative	31	29.2	
hsp60			
Positive	88	83.0	0.000*
Negative	18	17.0	
vacA			
Positive	101	95.3	0.000*
Negative	5	4.7	
vacAs1/s2			
Positive	101	95.3	0.000*
Negative	5	4.7	
vacAm1			
Positive	56	52.8	0.56
Negative	50	47.2	
vacAm2			
Positive	45	42.5	0.12
Negative	61	57.5	
N: Number			

We screened the 106 *H. pylori* positive gastric cancer patients and 106 healthy participants (control group), who were free of both *H. pylori* infection and gastric cancer, for TLR9 -1486T/C, rs187084 polymorphism by PCR-RFLP. The obtained frequencies of genotypes of TLR9 -1486T/C, rs187084 in

healthy group were all on line with Hardy–Weinberg equilibrium. PCR-RFLP assay analysis cleared that the frequency of CC genotype in gastric cancer patients (52.8%) was significantly higher than the control group (22.6%) with a P value of 0.045, whereas both TT and TC genotypes were not, as they recorded P values of 0.73 and 0.68, respectively as demonstrated in Table 5.

Table 5
Distribution of genotypes of TLR9 -1486T/C, rs187084 polymorphism in studied groups

Genotype frequency	Healthy study participants N=106		Gastric cancer patients N=106		OR	95% CI	X^2	P-value
	N	%	N	%				
TT	40	37.7	22	20.8	1.18	0.45-3.1	0.12	0.73
TC	42	39.6	28	26.4	1.2	0.49-2.88	0.16	0.68
CC	24	22.6	56	52.8	2.68	1.0-7.14	4.03	0.045*
Genotype frequencies are presented in the form of absolute numbers with percentages.								
OR: Odds ratio								
CI: Confidence interval								
N: Number								

The CC genotype of TLR9 -1486T/C, rs187084, when compared to TT + TC genotypes, demonstrated a significant relation with *H. pylori* strains carrying *sodB*, *hsp60* or *vacAm1* virulence genes (P = 0.021, P = 0.049 and P = 0.048 respectively). Whereas none of *cagA, vacA, vacAs1/s2* or *vacAm2* genes showed a significant association to CC genotype (P = 0.075, P = 0.88, P = 0.88 and P = 0.81 respectively), (Table 6).

Table 6
The relation between virulence genes of *H. pylori* and TLR9-1486T/C, rs187084 genotypes in gastric cancer patients

TLR9 -1486T/C, rs187084 genotype	CC N=56		TT + TC N=50		P-value
Virulence gene	**N**	**%**	**N**	**%**	
cagA	*55*	*98.2*	*41*	*82.0*	*0.075*
sodB	*53*	*94.6*	*22*	*44.0*	*0.021**
hsp60	*53*	*94.6*	*35*	*70.0*	*0.049**
vacA	*54*	*96.4*	*47*	*94.0*	*0.88*
vacAs1/s2	*54*	*96.4*	*47*	*94.0*	*0.88*
vacAm1	*35*	*62.5*	*21*	*42.0*	*0.048**
vacAm2	*19*	*33.9*	*26*	*52.0*	*0.81*
N: Number					

Discussion

Gastric cancer is a worldwide disease with a poor prognosis. *H. pylori* infection, through its pathogenic role, was recognized as an important carcinogen for gastric cancer (15). We have reported in our study a prevalence of *H. pylori* of 80.3% in patients with gastric cancer. This was in agreement with previous studies that reported a prevalence of *H. pylori* infection varying from 35% in developed countries up to 85% in developing ones with an estimated of 65-80% of gastric cancer attributable to *H. pylori* infection (31, 32). A slightly lower prevalence of *H. pylori* at 74.2% been reported by Wang and his colleagues (14). Nevertheless, a higher prevalence of *H. pylori* infection in gastric cancer patients been found by Enomoto et al. and Ezzat et al. at 98% and 100% respectively (33, 34). Such differences *in H. pylori* prevalence were attributed to the interaction between large diversity of factors including; genetic susceptibility and immune response of the host, presence of particular bacterial virulence factors, socioeconomic status and environmental factors (35).

Virulence traits of *H. pylori* have essential role in bacterial pathogenesis therefore they can be used to predict the infection outcome (1). In our study, the prevalence of *cagA* in *H. pylori* strains was 90.6% that was nearly similar to previous reports from Vietnam at 95%, (36), North America at 88% (37) and Sweden at 82% (21). However, a lower prevalence *of cagA* was reported by Ezzat et al. who detected the gene in 53.3% of *H. pylori* strains isolated from cancer patients (34).

The *vacA* gene has a mosaic structure with two main variation regions; the signal (s1 and s2), and the middle regions (m1 and m2) which determine the toxin vaculating activity (35). In our study, *vacA* gene

was detected in most of the isolated *H. pylori* strains (95.3%) which was consistent with other reports (1, 34). Also, we have reported the prevalence of *vacAm1* allele at 52.8% which was higher than that of *vacAm2* (42.5%). In line with our results, the *vacAm1* allele was reported to be commoner in Northern Asia while the *vacAm2* allele predominates in Southeast Asia which has a lower incidence of gastric cancer (16).

We detected *sodB* and *hsp60* genes in 70.8% and 83.0% of *H. pylori* strains respectively. In accordance with our findings, Ryberg and his colleagues have reported the prevalence of *sodB* and *hsp60* genes to be 72% and 82% respectively (21). However, a higher prevalence of *hsp60* gene (96.2%) has been reported by Amin et al. (1). such variations can be explained by; geographical diversity, different methodologies, genetic diversity and frequent genetic recombination among *H. pylori* strains. (1).

The host immune response plays an important role in determining the clinical prognosis of *H. pylori* infection. Therefore, polymorphisms in genes controlling the immune response can influence the risk to develop gastric cancer (14, 38). For TLR9 -1486T/C gene polymorphisms, we found that the frequency of CC genotype was significantly higher in gastric cancer patients than control group (P value= 0.045). Our findings suggest that CC genotype may have a role in modifying the immune response to *H. pylori* infection and subsequently promoting gastric carcinogenesis.

In line with our findings, Wang and his colleagues, reported that CC and TC genotypes of TLR9 -1486 were associated with a higher gastric cancer risk among the Chinese population. They also concluded that TLR9-1486 C allele carriers are associated with an increased risk along with poor prognosis of gastric cancer (14). Meanwhile, Susi et al. studied TLR9-1486 gene polymorphisms among Brazilian population and reported an association between CT and TT genotypes and increased risk to develop gastric cancer (11). On the other hand, both Zeng et al. and Liu et al. found no association between TLR9 -1486 T/C polymorphism and gastric cancer risk (39, 40). Such differences between reported results can be attributed to racial differences, environmental factors and diverse genetic backgrounds in various populations.

In a gene assay conducted by Tao et al., it was found that the C allele of TLR9 -1486T/C down-regulates the expression of TLR9 which leads to deficient immune recognition and signaling in response to *H. pylori* (41). Consequently, both innate and adaptive immune responses will be reduced which favor the development of chronic infection. As presence of chronic gastritis promotes the development of gastric cancer, individuals with CC genotype of TLR9 -1486T/C may have a higher risk for cancer, which can explain our findings.

Most studies have focused on either host or bacterial risk factors for developing gastric cancer, however in this study; we explored the relation between the studied virulence genes of *H. pylori* and genotypes of TLR9 -1486T/C (rs187084) polymorphism in gastric cancer patients. Since the CC genotype had a significantly higher frequency in gastric cancer patients than control group, we investigated its association with *H. pylori* virulence genes as compared to other genotypes. Interestingly, our findings has shown that CC genotype is significantly associated with *H. pylori* strains harboring *sodB, hsp60* or

vacAm1 virulence genes which further support the link between host genetics, bacterial virulence genes and gastric cancer. Such relation suggests a synergistic effect that may confer an increased risk to gastric cancer. Our findings can help clinicians to stratify patients according to their tendency to develop gastric cancer and to plan strategies for eradication of *H. pylori* infection. Further studies are recommended on larger number of cases and in more diverse populations to validate our results.

Conclusion

Patients with CC genotype of TLR9 -1486T/C (rs187084) are at higher risk for development of gastric cancer. In addition, the co-existence of CC genotype of TLR9 -1486T/C and *H. pylori* strains carrying *sodB*, *hsp60* or *vacAm1* virulence genes might have a synergistic effect promoting the development of gastric cancer.

Declarations

Ethical approval: This study was conducted in accordance with the Declaration of Helsinki besides the national and institutional standards. Its protocol was approved by the Mansoura faculty of medicine research board (R.21.01.1165).

Consent to participate:Informed written consent was received from all participants included in the study.

Funding: No funding was received for conducting this study.

Competing interests: No financial or non-financial interests to disclose.

Availability of data: Data will be made available on reasonable request

Author's contributions: Both YN and AS were the creators of the idea of the research. They both shared in designing the protocol to be carried out, conduction the samples processing procedures and also shared in writing the manuscript and analyzing resulting data. RNS shared in processing the samples besides writing and revising the manuscript. Both AMS and AS shared in performing surgical maneuvers and collecting required samples from included study members.

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