TLR4 and *NOD2/CARD15* Genetic Polymorphisms and their Possible Role in Gastric Carcinogenesis

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Abstract. *The aim of this study was to evaluate the role of TLR4 and NOD2/CARD15 genes in gastric carcinogenesis. Patients and Methods: We investigated the allelic frequencies of TLR4 (D299G and T399I) and NOD2/CARD15 (R702W, G908R, and L1007finsC) SNPs in 87 asymptomatic serologically H. pylori-positive individuals (Group I), in 63 patients with antrum-predominant gastritis (Group II) and in 60 patients with corpus-predominant gastritis or pangastritis (Group III). Results: There was significant difference in allelic frequencies of TLR4 D299G SNP in Group II (p=0.02; OR 2.97) as well as in Group III (p=0.001; OR 4.80). Significant difference of T399I SNP allele frequency was only found in Group III (p=0.009; OR 3.73). The allele frequencies of NOD2/CARD15 G908R and of L1007insC SNP were higher in Group III (p=0.003, OR 5.18; p=0.03; OR 3.66, respectively). Conclusion: TLR4 and NOD2/CARD15 genes are associated with high risk Group III patients and, therefore, they appear to play a role in gastric carcinogenesis.*

Although WHO has defined *Helicobacter pylori* as a class I gastric carcinogen (1), only a subset, 1% to 2% of infected individuals develops gastric malignancies. The risk for gastric cancer also increases in the presence of corpus-predominant gastritis as well as pangastritis (2). From a clinical point of view it is a major difficulty to identify those patients with *H. pylori* infection, who are at a higher risk of developing gastric cancer (3-5). Therefore, there is a need to identify patients who have these advanced changes in gastric mucosa.

Genetic variants that alter gene functions have been discovered for *TLR4* and *NOD2/CARD15,* which may underlie the variation in clinical outcomes from certain infections. Recently, it has been speculated that these genes are involved in handling the *H. pylori* attack (innate and adaptive immune responses) and that they mediate the resulting inflammation (6-8).

In this study, we investigated whether genetic polymorphisms in *TLR4* and *NOD2* were mainly associated with patients with corpus-predominant gastritis or pangastritis (high risk of gastric carcinoma) with respect to those with antrum-predominant gastritis or as asymptomatic serologically *H. pylori*-positive individuals (low risk of gastric carcinoma).

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Patients and Methods

Subjects. The control population consisted of serologically *H. pylori*-positive, but otherwise healthy volunteers (87 cases) who were without any gastrointestinal symptoms; they were medical students of Messina University School of Medicine. Endoscopy was not performed in the group of healthy individuals because, apart from ethical reasons, the probability of finding an active ulcer in patients without symptoms is very low. Consecutive patients referred for the investigation of dyspepsia to the endoscopic section of the University Hospital of Messina (Messina, Italy) were recruited from September 2007 to December 2008. All patients were from Eastern Sicily and Calabria (Southern Italy) and were tested for *H. pylori* infection. After the investigation, an endoscopic report was entered in a computer-based database. The following exclusions were applied: (i) a history of recent intake of acid suppressants, bismuth compounds, antibiotics or nonsteroidal anti-inflammatory drugs (within the preceding month); (ii) previous attempts at *H. pylori* eradication therapy; (iii) a history of previous gastric surgery; (iv) a history of chronic renal impairment The database was retrospectively analyzed for those patients with *H-pylori*-positive gastritis. Patients with gastric cancer were excluded, and 123 *H. pylori*-positive patients who fulfilled the predefined criteria were identified. All participants gave informed consent to the study, which was conducted in accordance with the Ethical Committee of the University Hospital.

H. pylori diagnosis. H. pylori infection was screened by histological examination, ^{13}C-urea breath test , or antibodies against *H. pylori*. Patients were diagnosed as *H. pylori*-positive if one or more of these diagnostic methods were positive.

Histopathology. The specimens for histological examinations were fixed in 10% buffered formalin, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin and with a

Table I. *Primers sequences and restriction enzymes used for genotyping TLR4 and NOD2/CARD15.*

Gene locus	SNPs		Sequence	Restriction enzyme
NOD2/CARD15	R702W	For	5' TTCAGATCACAGCAGCCTTC 3'	MspI
		Rev	5' CCCACACTGCAAAATGTCAAC 3'	
	G908R	For	5' AGCCACTGAAAACTCTTGG 3'	HhaI
		Rev	5' TCTTCACCTGATCTCCCCAA 3'	
	L1007finsC	For	5' CCTGCAGTCTCTTTAACTGG 3'	NlaIV
		Rev	5' CTTACCAGACTTCCAGGATG 3'	
TLR4	D299G	For	5' TTAGAAATGAAGGAAACTTGGAAAAG 3'	BsaBI
		Rev	5' TTTGTCAAACAATTAAATAAGTGATTAATA 3'	
	T399I	For	5' GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA 3'	HinfI
		Rev	5' CCTGAAGACTGGAGAGTGAGTTAAATGCT 3'	

modified Giemsa stain to detect the presence of curved rod-shape bacteria on the mucosal surface. All slides were re-evaluated by a experienced histopathologist (RAC), blinded to the endoscopic features and the results of the ^{13}C-urea breath test. The degree of inflammation, activity, atrophy, and metaplasia were assessed according to the updated Sydney system, and scored from 0 (normal) to 3 (marked). In these patients, the following parameters were calculated according to the histological examination: (i) Corpus –predominant gastritis: Higher degree of neutrophilic infiltration in the corpus compared to the antrum; (ii) Pangastritis: Equal degree of neutrophilic infiltration in the corpus and in the antrum; (iii) antrum-predominant gastritis: Higher degree of neutrophilic infiltration in the antrum compared to the corpus; (iv) Intestinal metaplasia: Absence or presence in any investigated specimen from antrum or corpus; (v) Severe atrophy: severe loss of glands.

All patients were subdivided into 3 groups: Group I (control group) (n=87 patients); Group II: duodenal ulcer phenotype characterized by an antrum-predominant gastritis (n=63 patients); Group III: gastric cancer phenotype characterized by a corpus-predominant or pangastritis (n=60 patients).

DNA extraction. Genomic DNA was isolated from 1 ml of peripheral blood anticoagulated with EDTA as previously described (9). DNA samples of the patients and control subjects were analyzed for the variants of *NOD2/CARD15* and *TLR4* genes by melting curve analysis.

Genotyping of the TLR4 polymorphisms. The two D299G and T399I SNPs of the *TLR4* gene were determined by PCR-RFLP. We performed PCR using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in a total volume of 25 μL. For D299G SNP, cycle conditions were an initial denaturation for 5 min at 95˚C, followed by 32 cycles of denaturing at 95˚C for 30 s, annealing at 51˚C for 30 s, primer extension at 72˚C for 30 s, followed by a final extension at 72˚C for 7 min. For T399I SNP, cycle conditions were an initial denaturation for 5 min at 95˚C, followed by 35 cycles of denaturing at 95˚C for 45 s, annealing at 55˚ C for 30 s, primer extension at 72˚C for 45 s, followed by a final extension at 72˚C for 7 min. The specific primers PCR and the restriction enzymes for each SNP are given in Table I. The amplified samples of *TLR4* gene D299G and T399I SNPs were digested at 37˚C, overnight, with the BsaBI and HinfI restriction enzymes (New England Biolabs, Ipswich, MA, USA), respectively. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA). Wild-type/mutant genotype was confirmed by automatic sequencing using the ABI-PRISM Big Dye™ Terminator v. 3.0 Cycle sequencing Ready Reaction Kit (Applied Biosystems, CA, USA). The sequencing products were purified using DyeEx Spin Kits (Qiagen) and visualized on an ABI-PRISM 310 Genetic Analyzer (Applied Biosystems, CA, USA).

Genotyping of the NOD2/CARD15 mutations. To detect the R702W, G908R, and L1007finsC mutations, we performed a polymerase chain reaction (PCR) using 0.5 U of Taq polymerase (Eurotaq, Euroclone Life Sciences Division, UK), 400 μmol/L dNTPs, and 0.1 μmol/L of each primer in a total volume of 25 μL. After an initial denaturation for 5 min at 95˚C, PCR was performed by 35 cycles of denaturing at 95˚C for 30 s, annealing at 65˚C for 40 s, primer extension at 72˚C for 30 s. The final extension was performed at 72˚C for 7 min. PCR reactions were carried out using a GeneAmp PCR system 2700 (Applied Biosystem, CA, USA). Genotyping of each SNP was performed by enzymatic digestion at 37˚C, overnight. After enzymatic digestion, the fragments were separated and visualized by gel electrophoresis (3% NuSieve® GTG agarose gel BMA, Rockland, ME, USA). The specific primers PCR and the restriction enzymes (New England Biolabs, Ipswich, MA) for each SNP are given in Table I. As previously described here, the results of enzymatic digestion were confirmed by DNA sequence analysis of representative samples of each SNP.

Statistical analysis. The Hardy-Weinberg equilibrium of the *TLR4* and *NOD2/CARD15* genes was assessed by χ^2 statistics. Differences in the allelic frequencies of *TLR4* and *NOD2/CARD15* genes among gastric cancer, gastric ulcer, duodenal ulcer, or gastritis patients and controls were determined by the χ^2 test using a 3×2 contingency table. The individual genotype, and allele frequencies, was analysed using a 2×2 contingency table with chi-square test, and the odds ratio (OR) with 95% confidence interval (CI) were calculated. A *p*-value <0.05 was considered statistically significant. The computer program STATA 9 was used for statistical analysis.

Results

Allele frequencies of TLR4 D299G and T399I SNPs. The allelic distributions of the *TLR4* gene D299G and T399I SNPs among the groups of the study are listed in Table II. The genotype distributions for the D299G and T399I

Table II. *Allele frequencies of TLR4 SNPs D299G and T399I in Group I, Group II and Group III patients.*

Polymorphism	Control group I* (n=87)	Allelic frequency (%)	Group II (n=63)	Allelic frequency (%)	^1P-Value	OR (95% CI)	Group III (n=60)	Allelic frequency (%)	^2P-Value	OR (95% CI)
D299G										
Wild-type	80 (91.9%)	4.0%	50 (79.4%)	10.0%	0.02	2.97 (1.13-7.73)	42 (70.0%)	15.0%	0.001	4.80 (1.93-12.35)
Heterozygous	7 (8.1%)		13 (20.6%)				18 (30.0 %)			
Mutant homozygous	0 (0%)		0 (0%)				0 (0%)			
T399I										
Wild-type	81 (93.1%)	3.4%	57 (90.4%)	4.7%	n.s.	1.42 (0.45-4.40)	57 (95.0%)	10.8%	0.009	3.73 (1.36-10.14)
Heterozygous	6 (6.9%)		6 (9.6%)				13 (5.0%)			
Mutant homozygous	0 (0%)		0 (0%)				0 (0%)			

*Serologically *H. pylori*-positive patients, but without any symptoms; no patients homozygous for D299G and T399I SNPs were found in this study population. ^1P, control group I *vs*. Group II patients; ^2P, control group I *vs*. Group III patients.

Table III. *Allele frequencies of NOD2/CARD15 SNPs in Group I, Group II and Group III patients.*

Polymorphism	Control group I * (n=87)	Allelic frequency (%)	Group II (n=63)	Allelic frequency (%)	^1P-Value	OR (95% CI)	Group III (n=60)	Allelic frequency (%)	^2P-Value	OR (95% CI)
R702W										
Wild-type	84 (96.5%)	1.7%	61 (96.8%)	1.5%	n.s.	0.92 (0.17-4.75)	58 (96.6%)	1.6%	n.s.	1.16 (0.22-6.07)
Heterozygous	3 (3.5%)		2 (3.2%)				2 (3.4%)			
Mutant homozygous	0		0				0			
G908R										
Wild-type	83 (95.4%)	2.3%	55 (87.3%)	6.3%	n.s	3.01 (0.91-9.88)	48 (80.0%)	10.0%	0.003	5.18 (1.65-16.09)
Heterozygous	4 (4.6%)		8 (12.7%)				12 (20.0%)			
Mutant homozygous	0		0				0			
L1007finsC										
Wild-type	83 (95.4%)	1.7%	60 (85.0%)	2.3%	n.s.	1.03 (0.25-4.31)	51 (95.5%)	7.5%	0.03	3.66 (1.13-11.80)
Heterozygous	4 (4.6%)		3 (15.0%)				9 (4.5%)			
Mutant homozygous	0		0				0			

*Serologically *H. pylori*-positive patients, but without any symptoms; no patients homozygous for R702W, G908R and L1007finsC SNPs were found in this study population. ^1P, control group I *vs*. Group II patients; ^2P, control group I *vs*. Group III patients.

polymorphisms were not different from those expected by Hardy-Weinberg equilibrium for all studied groups.

Allele frequencies of TLR4 D299G SNP. In Group II patients, the allele frequency of D299G SNP was significantly higher (10%) than that in group I (4%) (p=0.02; OR, 2.97; 95% CI, 1.13-7.73). A significant difference in the allelic frequencies of this SNP was found between Group I (4%) and Group III (15%) (p=0.001; OR, 4.80; 95% CI, 1.93-12.35). No significant difference of the T399I SNP allelic frequencies was found between Group I (3.4%) and Group II (4.7%) (p=n.s.; OR, 1.42; 95% CI, 0.45-4.40). In Group III patients, the allelic frequency of this SNP was significantly higher (10.8%) than in Group I (3.4%) (p=0.009; OR, 3.73; 95% CI, 1.36-10.14). No homozygous carriers of the two *TLR4* gene SNPs were found in any group study. The *TLR4* allelic frequencies were in Hardy-Weinberg equilibrium in all patients and in control subjects.

Allele frequencies of NOD2/CARD15 gene R702W, G908R, and L1007finsC SNPs. In Group II patients, the allelic frequency of the R702W SNP (1.5%) was not significantly different from that of Group I (1.7%), p=n.s.; OR, 0.92; 95% CI, 0.17-4.75). No significant difference was found between

the allelic frequencies of the Group I (1.7%) and Group III (1.6%) (p=n.s. OR, 1.16; 95% CI, 0.22-6.07). In Group II patients, the allelic frequency of the G908R SNP (6.3%) was not significantly different from that of Group I (2.3%) (p=n.s; OR, 3.01; 95% CI, 0.91-9.88). Significant difference in the allelic frequencies of this SNP was found between the Group I (2.3%) and the Group III patients (10.0%) (p=0.003; OR, 5.18; 95% CI, 1.65-16.09). In Group II patients, the allelic frequencies of the L1007insC SNP (2.3%) was not significantly different from that of Group I (1.7%) (p=n.s.; OR, 1.03; 95% CI, 0.25-4.31). Significant difference was found in the allelic frequencies of this SNP between the Group I (1.7%) and the Group III patients (7.5%) (p=0.03; OR, 3.66; 95% CI, 1.13-11.80). No homozygous carriers of the three *NOD2/CARD15* gene SNPs were found in the study groups. The *NOD2/CARD15* allelic frequencies were in Hardy-Weinberg equilibrium in all patients and in controls (Table III).

Discussion

A large prospective study from Japan has recently shown that only patients with *H. pylori* infection develop gastric cancer (2). A closer look at the data reveals that certain histopathological findings increased the risk dramatically. In fact, the presence of intestinal metaplasia (RR 6.4), severe atrophy (RR 4.9), pangastritis (RR 15.6), and most strikingly, corpus-predominant gastritis (RR 34.5) is associated with an increased risk for gastric cancer (2).

There are several limitations in previous studies regarding association between *TLR4* and *NOD2/CARD15* genetic polymorphisms and precursor lesions of gastric carcinomas. In fact, most studies are based on the old hypothesis of an atrophy–metaplasia–dysplasia–carcinoma sequence in the stomach. In other words, many authors considered intestinal metaplasia and gastric atrophy as the only precancerous conditions and did not mention corpus-predominant gastritis and pangastritis, which are strong risk factors for gastric carcinomas instead (2). In our study a comparative topographic analysis of the grade and activity of *H. pylori* gastritis in the antrum and corpus was performed. Furthermore, we also evaluated whether some *TLR4* and *NOD2/CARD15* genetic SNPS can be associated with high risk precancerous conditions such as pangastritis and corpus-predominant gastritis (Group III) in respect to antrum-predominant gastritis (low risk) (Group II) and controls.

TLR4 D299G and T399I SNPs are located in the coding sequence and affect TLR4 extracellular domain. Several studies found that the carriers of these SNPs are hyporesponsive to LPS challenge by either disrupting transport of TLR4 to the cell membrane or by impairing ligand binding or protein interactions (10, 11). Therefore, the potential mechanism by which the *TLR4* polymorphisms increase the risk of gastric cancer and its precursors may lie in the nature of the host overall response to the *H. pylori* lipopolysaccharide attack (10-12). Failure to handle the invasion by appropriately recognising and activating the necessary pathways may lead to an exaggerated inflammatory response (13, 14). It is relevant to the entire multistage process of gastric carcinogenesis, which starts with *H. pylori* colonization of the gastric mucosa (15-18). Accordingly, our data show that *TLR4* T399I polymorphism was significantly associated with Group III patients (OR 3.07); *TLR4* D299G was associated with Group II patients (OR 2.97) and with Group III patients (OR 4.80) with respect to Group I patients. In our study, we also analyzed the influence of *NOD2/CARD15* gene R702W, G908R, and L1007finsC SNPs on susceptibility to gastric cancer. *NOD2/CARD15* is a member of the NOD-like receptor gene family implicated in programmed cell death and immune responses to intracellular bacteria. It has been reported that the expression of NOD2 is up-regulated under inflammatory conditions in epithelial cells of the gastric mucosa (19). Common mutations in the *NOD2/CARD15* gene have already been associated with an increased odds ratio of developing cancer of the bowel (20) or breast (21), and with the increased incidence of malignancy in Crohn's disease (22) and an overall increase in malignancies at other sites including breast, lung, larynx and ovary (23). Experimental evidence suggests that NOD2/CARD15 protein is involved in either the recognition of bacterial polysaccharides and/or activation of NFκB. Loss of NOD2/CARD15 function is predicted to result in excessive NFκB activity, thereby altering the risk of disease by contributing to the inflammation–dysplasia–carcinoma sequence (24). In our study, G908R and L1007finsC polymorphisms were significantly associated with Group III patients (OR 5.18 and 3.66, respectively). There was no association between R702W polymorphism and the three groups of patients.

In conclusion, our preliminary results suggest that *TLR4* and *NOD2/CARD15* genes are biologically plausible disease-modifying factors. If confirmed in multiple populations, these *TLR4* and *NOD2/CARD15* genetic polymorphisms, in conjunction with others, could be used to identify patients at greater risk for gastric cancer, who may need closer monitoring.

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