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Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms in gastric cancer of intestinal and diffuse histotypes

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Summary

In the present study we investigated the potential role of Toll-like receptor 4 (TLR-4) Asp299Gly and Thr399Ile polymorphisms as risk factors in the development of gastric cancer. TLR-4 Asp299Gly and Thr399Ile polymorphisms were investigated in 171 Italian patients with sporadic gastric cancer and in 151 controls. Unconditional regression (odds ratio and 95% confidence intervals) were used to investigate the association of the studied polymorphisms with gastric cancer. TLR-4 Thr399Ile polymorphism is linked with an increased susceptibility to gastric cancer ($P = 0{\cdot}023$ and hazard ratio = 3·62). No significant association for TLR-4 Asp299Gly polymorphism was found. In the subgroup of patients with intestinal-type gastric cancer, a significant risk of gastric cancer was associated with TLR-4 Thr399Ile genotype ($P = 0{\cdot}006$). Our results demonstrated that TLR-4 Thr399Ile polymorphism is linked with an increased susceptibility to gastric cancer. An increased risk for intestinal gastric cancer in carriers of the *TLR4* Thr399Ile allele was observed. Future epidemiological studies should consider the possible interactions between proinflammatory genotypes (such as TLR and interleukin-1R polymorphisms) and other risk factors for cancer such as dietary habits and/or exposure to environmental carcinogens.

Keywords: gastric cancer, incidence, polymorphisms, TLR-4

Introduction

The innate immune response is the primary first line of defence against invading pathogens. Toll-like receptors (TLRs) are a family of receptors which play a pivotal role in sensing a wide range of pathogens, including bacteria, fungi and viruses. These receptors function as pathogen recognition receptors, recognizing pathogen-associated molecular patterns (PAMPs) which are unique to microbes [1].

TLRs are characterized by the presence of an extracellular leucine-rich domain and an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain. They recognize different ligands, such as PAMPs or the 5′cytosine-phospho-guanine island containing DNA. TLR signalling is initiated by a distinct set of pathogens that activate downstream transcription factors by different adaptor proteins and lead to cytokine production through pathways that are not, as yet, well known [1,2].

A total of 10 TLRs are expressed in humans. Among TLRs, TLR-4 allows lipopolysaccharide (LPS) recognition which requires CD14 and MD2 as co-receptors. Once this pathway is triggered the adaptor molecules (MAL, MyD88) are recruited to initiate downstream signalling: activation of the I-kB complex, translocation of nuclear factor kappa B (NF-kB) factor and production of proinflammatory cytokines [tumour necrosis factor-α, IL-1, IL-6, IL-8, IL-12] [3,4]. Moreover, the interaction between TLR-4 and antigen-presenting cells (APCs) results in the up-regulation of co-stimulatory molecules such as CD40, CD80 and CD86 on APCs [5].

Dysregulation of TLR signalling has been implicated in the pathogenesis of different diseases such as sepsis, malaria, candidiasis, experimental autoimmune encephalomyelitis, systemic lupus erythematosus, diabetes, cardiomyopathy, asthma and chronic obstructive pulmonary disease [6]. Moreover, several single nuclear polymorphisms (SNPs) within individual TLRs have been identified and linked to a disease condition. The presence of gene polymorphisms, particularly those identified in TLR-2 and TLR-4, has been associated with an increased susceptibility to infection/sepsis, or with atherosclerosis or asthma [6].

Human TLR-4 is located on chromosome 9q32–q33, contains four exons and it is highly expressed on lymphocytes, monocytes, polymorphonuclear leucocytes and splenocytes

[7]. Polymorphisms in TLR-4 have already been studied. Among them are: A+896G (SNP ID: rs 4986790) and C+1196T (SNP ID: rs 4986791). These SNPs are located in the coding sequence, affect TLR-4 extracellular domain and result in amino acid exchanges: an aspartic acid for a glycine at position 299 (Asp299Gly) and a threonine for an isoleucine at position 399 (Thr399Ile). Individuals possessing a co-segregating polymorphism in TLR-4 (Asp299Gly and Thr399Ile) are hyporesponsive to LPS and are more susceptible to Gram-negative bacterial infections [8,9].

The TLR activation plays a role in initiating the inflammatory response. As a consequence, a dysregulation of TLR signalling may contribute to an unbalanced ratio between pro- and anti-inflammatory cytokines and thus to a higher risk of developing chronic inflammatory diseases and cancer [10]. It is well known that chronic inflammation plays an important role in promoting several human cancers [11,12].

The mechanisms by which TLR polymorphisms impact on cancer risk is demonstrated in gastric and prostate cancer, two malignancies in which chronic inflammation pathogenesis is the underlying pathological event [13].

The presence of SNPs in the TLR family is associated with enhanced prostate cancer risk [14,15].

Moreover, because of the established role of *Helicobacter pylori* (HP) infection in the aetiology of gastric cancer [16], recent studies have been focused upon the role of TLRs in the inflammatory response of gastric mucosa against HP infection. The results obtained are controversial: while some studies have reported that TLR-4 is essential for the immune response established during HP-related gastritis [17,18], others have emphasized the central role of TLR-2 [19,20]. Otherwise, it has been shown that HP enhances gastric ulcer and cancer risk, particularly in those subjects affected by chronic mucosal inflammation [16].

The presence of a polymorphism, such as those in the TLR-4 Asp299Gly and Thr 399Ile, affecting genes involved in cytokine expression could represent an additional genetic risk factor to develop gastric cancer [12,21–24].

Furthermore, the association between IL-1 receptor (IL-1R) gene polymorphism and enhanced gastric cancer risk has been demonstrated [20,21,25]. Of note, TLRs are characterized by a TIR intracellular domain with high homology to IL-1R. For all these reasons it is conceivable to study TLRs polymorphisms as possible susceptibility factors in the development of gastric cancer.

In the present case–control study we investigated the potential role of TLR-4 Asp299Gly and Thr399Ile polymorphisms in Italian gastric cancer patients.

Materials and methods

Study subjects

Peripheral blood samples from patients with sporadic gastric cancer and healthy controls were collected in high-risk areas for disease in Central Italy between September 2005 and January 2006. The inclusion criteria for gastric cancer patients and healthy controls were: Caucasian ethnicity, residency in one of the five different geographical areas of Central Italy, lack of previous personal and family history of cancer. Current and former blood donors were used as healthy controls and they were identified through the pools of blood donors available at each participating institution. Controls were selected randomly with frequency matching to cases by age ($\pm$4 years) and gender. Before study inclusion, eligible patients and healthy controls were interviewed about personal/family medical history, education, tobacco smoking and alcohol intake. Pedigrees were traced back for at least three generations and laterally to second- and third-degree relatives. The threshold of 20 g/day of alcohol intake was estimated as approximately two cans of beer, two glasses of wine or two shots of spirit. The diagnosis of gastric cancer was confirmed histologically by two independent pathologists. The histological types characterized were: intestinal type adenocarcinoma (tumour cells describe irregular tubular structures, harbouring pluristratification, multiple lumens, reduced stroma) and diffuse type adenocarcinoma (tumour cells are discohesive and secrete mucus which is delivered in the interstitium, producing large pools of mucus/colloid; it is poorly differentiated). The ethical requirements were verified by the internal review boards and all participants gave their written informed consent.

Analysis of TLR-4 Asp299Gly and Thr399Ile polymorphisms

Genomic DNA was extracted from peripheral blood lymphocytes [26]. Allele-specific polymerase chain reactions (PCRs) were used to detect TLR-4 Asp299Gly and Thr399Ile polymorphisms in DNA samples extracted from 5 ml of total blood samples, as reported by Lorenz *et al.* [27]. Briefly, a fragment containing the repeats was amplified using the following primers: Asp299Gly: AF 5′-ATTAGCATACTTAG ACTACTACCTCCATG-3′; AR 5′-GATCAACTTCTGAAAA AGCATTCCCAC-3′; and Thr399Ile: TF 5′-GTTGCTGT TCTCAAAGTGATTTTGGGAGAA-3′ and TR 5′-ACCTG AAGACTGGAGAGTGAGTTAAATGCT-3′. PCR products were digested with NcoI and Hinf I restriction enzymes and analysed by 3% agarose gel electrophoresis. TLR-4 polymorphisms were confirmed by DNA sequencing of both strands to verify the segregation patterns of TLR-4 399 and TLR-4 299. Homozygotes and heterozygotes could be distinguished using the current genotyping method [27].

Statistical analysis

Analysis of the association between gastric cancer and risk factors involved in the presence of TLR-4 polymorphisms was performed and assessed by logistic regression analysis using STATA statistical software (Stata Corporation, College

Table 1. Characteristics of the study population.

	Gastric cancer cases			
	Controls ($n = 151$)	All ($n = 171$)	Intestinal ($n = 87$)	Diffuse ($n = 84$)
Age (years)	56 (30–80)	60 (34–82)	60 (38–80)	60 (38–81)
Gender*				
Male	87 (58%)	99 (58%)	50 (58%)	47 (56%)
Female	64 (42%)	72 (42%)	37 (42%)	37 (44%)
Educational level**				
≤ 8 years	78 (52%)	94 (55%)	49 (56%)	49 (59%)
> 8 years	73 (48%)	77 (45%)	38 (44%)	35 (41%)
Smoking status***				
Current smokers	53 (35%)	55 (32%)	39 (45%)	30 (36%)
Non-smokers	98 (65%)	116 (68%)	48 (55%)	54 (64%)
Alcohol intake****				
Current drinkers	59 (39%)	68 (40%)	36 (41%)	26 (31%)
Non-drinkers or < 20 g/day	92 (61%)	103 (60%)	51 (59%)	58 (69%)

*$P = 0{\cdot}451$; **$P = 0{\cdot}956$; ***$P = 0{\cdot}639$; ****$P = 0{\cdot}735$.

Station, TX, USA). Fisher's and χ^2 tests with Yates' correction were performed using the statistical program EPI6 (Center for Disease Control and Prevention, Atlanta, GA, USA). To evaluate the risk associated with the presence of specific polymorphisms, unconditional regression analysis to define the hazard ratio (HR) with 95% confidence interval (CI) was adopted adjusted for age, sex, alcohol intake and smoking status.

Results

Study population characteristics

The study population consisted of 171 gastric cancer patients and 151 controls. The median age of the patients was 60 years (range 34–82 years) and for the controls was 56 years (range 30–80 years) while the male/female ratio was 99/72 and 87/64 among patients and controls, respectively. Based on Lauren's classification, the gastric carcinomas were either of intestinal phenotype in 87 patients (51·2%) or of diffuse histotype in 84 patients (48·8%), as shown in Table 1. Educational levels and the amounts of alcohol intake and tobacco smoking were comparable between patients and controls. The genotypes observed were in Hardy–Weinberg equilibrium.

The TLR-4 polymorphisms and gastric cancer risk

The TLR-4 polymorphisms, detected using allele-specific PCRs, were found in 28 of 171 (16·4%) gastric cancer patients and in 15 of 151 (9·9%) controls, as shown in Table 2.

Among the gastric cancer patients, 12 of 171 (7%) had a TLR-4 Asp299Gly polymorphism and 16 of 171 (9·3%) a TLR-4 Thr399Ile polymorphism, while nine of 171 (5·2%) showed both TLR-4 Asp299Gly and Thr399Ile polymorphisms (Table 2); among healthy controls, TLR-4 polymorphisms were detected in 15 of 151 (9·9%), with 11 of 151 (7·3%) having a TLR-4 Asp299Gly polymorphism, four of 151 (2·6%) a TLR-4 Thr399Ile polymorphism and two of 151 (1·32%) having both TLR-4 Asp299Gly and Thr399Ile polymorphisms (Table 2). Except for one homozygous patient, all the TLR-4 polymorphisms found were heterozygous both in patients and controls.

Statistical analysis revealed a significant correlation between the presence of a TLR-4 Thr399Ile polymorphism and the incidence of gastric cancer [$P = 0{\cdot}023$ by Fisher's test and HR = 3·62 (1·27–6·01); Table 3]. Similar results were obtained using a χ^2 test ($\chi^2 = 5{\cdot}156$; Table 3). There was no significant correlation between the presence of a TLR-4 Asp299Gly polymorphism and the risk of gastric cancer [$P = 0{\cdot}832$ by Fisher's test and HR = 0·97 (0·37–1·143); Table 3].

Table 2. Polymorphisms of Toll-like receptor 4 (TLR-4) gene in gastric cancer patients and controls.

TLR-4 polymorphism	Genotype frequencies	Gastric cancer	Controls
Number		171	151
Asp(299Gly)	GG	1 (0·58%)	0 (0%)
	AG	11 (6·4%)	11 (7·3%)
	AA	159 (93%)	140 (93%)
Thr(399)Ile	TT	1 (0·58%)	0 (0%)
	CT	15 (8,8%)	4 (2·6%)
	CC	155 (91%)	147 (97%)

Table 3. Logistic regression model (outcome variable: gastric cancer presence).

Covariate analysis	HR	95% CI	χ^2	P
Asp299Gly	0·97	0·37–1·143	0·045	0·832
Thr399Ile	3·62	1·27–6·01	5·156	0·023

CI, confidence interval; HR, hazard ratio.

Table 4. Correlation between polymorphisms and the incidence of different histotypes of gastric cancer.

	HR	95% CI	χ^2	*P*
Diffuse type				
Asp299Gly	0·55	0·29–1·312	0·713	0·398
Thr399Ile	1·85	0·712–2·131	0·258	0·612
Intestinal type				
Asp299Gly	1·37	0·576–1·751	0·333	0·564
Thr399Ile	5·38	1·652–8·145	7·445	0·006

CI, confidence interval; HR, hazard ratio.

The frequency of the TLR-4 Thr399Ile carriers was significantly higher in patients with intestinal gastric cancer than controls ($P = 0·006$). The HR for TLR-4 Thr399Ile carriers was 5·38 (95% CI: 1·652–8·145) in the intestinal gastric cancer group (Table 4). No correlation with gastric cancer risk was observed for TLR-4 Asp299Gly in the intestinal histological subgroup ($P = 0·564$). No correlation with gastric cancer risk was observed for TLR-4 Asp299Gly and TLR-4 Thr399Ile genotypes in the diffuse histological subgroup ($P = 0·398$ and $P = 0·612$, respectively) (Table 4).

Discussion

The TLRs are involved in innate immunity defence against microorganisms. They are members of a larger superfamily that includes the IL-1Rs. TLR pathway activation is essential for the innate immune response against pathogens, but impaired signalling is involved in the pathogenesis of autoimmune, chronic inflammatory and infectious diseases [2,3]. Moreover, TLR signalling activation results in proinflammatory cytokine production at local or systemic levels.

Several SNPs within individual TLRs have been identified. With regard to TLR-4, two major polymorphisms have been described (Asp299Gly and Thr399Ile) [8,9]. Different studies have reported a link between Asp299Gly and Thr399Ile polymorphisms and gastric inflammation, as well as mucosa lesions related to HP infection [17–20]. Moreover, a correlation between *H. pylori* infection, precancerous lesions of the stomach, is reported [16].

Subjects with TLR-4 polymorphisms have an increased risk of severe inflammation and subsequent development of hypochlorhydria and gastric atrophy, which are regarded as the most important precancerous abnormalities [13]. Recent epidemiological and genetic association studies suggest that chronic inflammation may play an important role in the development of several human cancers, such as gastric cancer. Furthermore, genetic polymorphisms, such as TLR-4 polymorphisms Asp299Gly and Thr399Ile, can lead to persistent inflammation, suggesting the possibility of increased genetic cancer susceptibility in subjects carrying TLR-4 polymorphisms [18,19].

Currently, there are no conclusive data on TLR-4 Thr399Ile and Asp299Gly allele frequencies in Italian patients with gastric cancer. Based on this and the above-mentioned clinical association, we focused our study specifically upon TLR-4 Asp299Gly and Thr399Ile polymorphisms in this subset of patients.

Our results demonstrated that the TLR-4 Thr399Ile polymorphism is linked with an increased susceptibility to gastric cancer. In fact, the TLR-4 Thr399Ile polymorphism showed a significant correlation with the incidence of gastric cancer ($P = 0·023$ by Fisher's test and HR = 3·62), while the TLR-4 Asp299Gly polymorphism did not ($P = 0·832$ by Fisher's test and HR = 0·97). In addition, we observed an increased risk for intestinal gastric cancer in carriers of the TLR-4 Thr399Ile allele. To the best of our knowledge, this is the first report on the association between the TLR-4 Thr399Ile polymorphism and gastric cancer risk.

El-Omar *et al.* [13] proposed that subjects with a proinflammatory genetic phenotype, based on a combination of markers from adaptive IL-1β and the innate TLR immune response, respond to *H. pylori* infection by creating an environment within the stomach that is chronically inflamed and with reduced acidity. This environment is conductive to the growth of non-HP bacteria leading to sustained inflammation, thus maintaining the pro-neoplastic drive.

We evaluated the distribution of infection with *H. pylori* both in patients and controls, but we found a similar distribution in the two groups without any statistically significant difference.

Moreover, no correlation was found between the appearance of disease and HP infection as well as between the presence of TLR-4 polymorphisms and HP infection. However, these findings do not challenge the aetiological role of HP in the pathogenesis of gastric cancer and they should be interpreted cautiously. It is probable that the lack of statistical correlation with HP could be explained by the size of the population. Moreover, patient stratification into subgroups according to gastric cancer histological type and site (cardiac and non-cardiac) could provide interesting findings, but the population size was not high enough to support a statistically significant analysis.

It is conceivable to hypothesize that a direct effect of the TLR induced proinflammatory cytokines on gastric mucosa, and their interactions with environmental carcinogens could represent one of the mechanisms by which TLR-4 polymorphisms increase susceptibility to gastric cancer. Future epidemiological studies should consider the possible interactions between proinflammatory genotypes (such as TLR and IL-1R polymorphisms) and other risk factors for cancer, such as dietary habits and/or exposure to environmental carcinogens. The non-obese diabetic (NOD) (nucleotide-binding oligomerization domain) proteins NOD1 and NOD2 have been shown to play important roles in innate immunity as sensors of microbial components derived from bacterial peptidoglycan [28]. Polymorphisms in NOD2 (CARD15) are associated with ileal and ileocolonic Crohn's disease [29]; their role is complex and incompletely

understood. For these reasons, future epidemiological studies should consider possible interactions between inflammatory genotypes such as NOD2 polymorphisms and risk of gastric cancer.

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