Tumor Necrosis Factor Alpha Extended Haplotypes and Risk of Gastric Carcinoma

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Abstract

The tumor necrosis factor α (*TNF*A)-308*A allele has been found to confer an increased risk of gastric carcinoma. Inconsistency in risk estimates across populations lead us to hypothesize about the presence of an alternative causal locus in the same chromosomal region. A suitable approach is to determine the tumor necrosis factor haplotypic structure in order to clarify whether the association between the *A allele and the increased risk of gastric carcinoma is etiologic or secondary to linkage disequilibrium. Firstly, we assessed the association between the *TNFA*-308G>A polymorphism and the risk of gastric carcinoma in a population from Northern Portugal (508 gastric carcinoma patients, 713 controls); secondly, we genotyped five microsatellite loci (TNFa, b, c, d, e) flanking the *TNF*A-308G>A locus to establish the haplotypic structure associated with this single-nucleotide polymorphism in cases (122 patients) and controls (169 individuals). We found a significant association between the *A allele and increased risk of gastric carcinoma (odds ratio, 1.7; 95% confidence interval, 1.3-2.2) confirming previous results in our population. Regarding the *A allele–associated haplotypes, the most relevant difference was found for the H1A haplotype present in 33.1% of the cases and 12.5% of the controls. We also observed haplotypes associated with the *A allele that were found only in cases or controls. A population differentiation test showed that the gastric carcinoma and the control groups were significantly different for the *A allele haplotypic structure. This suggests that the association between the *TNFA*-308G>A polymorphism and increased risk of gastric carcinoma is dependent on linkage disequilibrium with an as yet unidentified locus. (Cancer Epidemiol Biomarkers Prev 2008;17(9):2416–20)

Introduction

Gastric carcinoma is one of the most common malignancies worldwide. Although decreasing in incidence in the past 50 years, it remains the second most common cause of cancer-related death. Estimates predict 1.1 million new cases in the year 2010 (1, 2). The *Helicobacter pylori* bacterium colonizes the human stomach and is the most recognized and well-known etiologic risk factor for gastric carcinoma (3). It possesses a unique array of features that makes it highly adapted to this ecological niche and causes a long-term infection of the gastric mucosa. The strong inflammatory response by the human host usually does not eradicate the bacteria then causing damage on the gastric epithelial cells (4). The infection first induces chronic superficial (non-atrophic) gastritis, which can progress through chronic atrophic gastritis, intestinal metaplasia, and dysplasia towards gastric carcinoma (5).

The tumor necrosis factor (TNF) α is a pleiotropic cytokine mostly produced by activated monocytes and macrophages, which play a key role in the inflammatory response (6, 7). Thus, it is a strong candidate for mediating the risk of developing immunologically related malignant disease. The *TNFA* gene, which encodes the TNFα cytokine, is located within the human leukocyte antigen class III region of the major histocompatibility complex on chromosome 6 (section 6p21). This region is highly polymorphic and contains a number of immune response genes such as lymphotoxin α (also known as *TNFB*) and the lymphotoxin β (also known as *TNFC*).

Several single-nucleotide polymorphisms (SNP) have been identified in the *TNFA* gene, mainly in the 5′-promoter region. Five microsatellites (TNFa, b, c, d, and e), occurring together with those SNPs in extended haplotypes, have also been described (8-10). Many studies have investigated the relation between TNF polymorphisms and the susceptibility to or outcome of various infectious (11, 12), inflammatory (13, 14), and neoplastic diseases (15-17). Although several promoter polymorphisms have been analyzed, most studies have focused on the *TNFA*-308G>A (rs1800629). Since the first reports of *TNFA* association with gastric carcinoma (18-22), the *TNFA*-308*A allele has emerged as an

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important candidate accounting for the increased risk of gastric carcinoma development (23, 24). Nevertheless, outcomes of the numerous studies on whether polymorphisms in or near *TNF* genes do underlie these associations are conflicting. While *TNFA* alleles have been associated with the genetic predisposition to differential secretion of TNFα, the functional assays described in the literature remain contradictory (25, 26).

Numerous association studies of disease susceptibility target candidate genes based only on their putative functionality. The polymorphisms are then analyzed independently of the genetic structure of the chromosome where they are located. An alternative strategy is the establishment of the haplotypic structure inferred from allelic variants at several loci. The main advantage of this alternative is that, with this genetic information, gene-disease association studies can be performed without prior assumptions on the functional relevance of specific variants.

Several studies have described associations between TNF microsatellites and infectious diseases as well as cancer. However, none have studied TNF microsatellites extended haplotypes and the risk of gastric carcinoma. In the present investigation, we performed a case-control study for the *TNFA*-308G>A polymorphism in order to determine the association with the risk of gastric carcinoma development. The subsequent aim was to establish the haplotypic structure associated with the *TNFA*-308G>A polymorphism in both cases and controls by genotyping five polymorphic microsatellite loci (TNFa, b, c, d, and e) flanking the *TNFA* gene over a 20 kb region. Our results indicate that the detected association between the pro-inflammatory *TNFA*-308*A allele and the increased risk of gastric carcinoma is at least partially influenced by linkage disequilibrium with an as yet unidentified *locus*.

Materials and Methods

Study Population. A total of 1,221 subjects from the North of Portugal were enrolled in this study, comprising gastric carcinoma patients (n = 508) and controls (n = 713). The control group consisted of healthy blood donors (n = 318) and a representative sample of the non-institutionalized adult population of Porto (n = 395), Portugal, selected during the assembling of the EpiPorto cohort (mean age, 37 y; median age, 35 y; range,18-64 y; male:female ratio, 1.7:1). A detailed description of the selection procedures and participants was published previously (27). Patients with gastric carcinoma (mean age, 56 y; median age, 58 y; range, 26-90 y; male:female ratio, 1.4:1) had been diagnosed and treated by tumor resection at Hospital S. João, Porto, Portugal.

Genotyping. Genomic DNA from the blood samples of control individuals and from gastric carcinoma patients was isolated using standard proteinase K digestion and phenol/chloroform extraction. Genotyping of the *TNFA*-308G>A SNP was done by the 5′-nuclease PCR assay (TaqMan). TaqMan (SNP Genotyping Assays) primers, probes, and amplification conditions are available upon request. In a subset of 50 samples, the genotypes were confirmed by direct sequencing. The five TNF-linked microsatellites (TNFa, b, c, d, and e; Fig. 1) were genotyped using PCR and GeneScan in a total of 291 individuals (169 controls and 122 gastric carcinoma patients randomly selected from the total sample) and following methods previously described (28).

Statistical Analysis. Evidence for deviation from Hardy-Weinberg equilibrium of alleles at individual loci was assessed by exact tests using GENEPOP (ref. 29; ftp://ftp.cefe.cnrsmop.fr/pub/pc/msdos/genepop/). Differences in the proportion of the *TNFA*-308G>A genotype between cases and controls was assessed using the χ^2 test. Crude odds ratios (OR) with 95% confidence intervals (95%′CI) were estimated by unconditional logistic regression analysis using SPSS (SPSS for Windows, Rel. 14.0.0. 2005, SPSS Inc.). Differences were considered to be significant at a P <0.05. All statistical tests were two-sided. Haplotypes defined by the association of alleles from the *TNFA*-308G>A SNP and the five microsatellite loci were inferred by the maximum likelihood approach implemented in the ARLEQUIN v.2.0. software package (30). Population differentiation was tested using ARLEQUIN v.2.0. Haplotypes associated with the *TNFA*-308*G allele were designated as follows: H1G-TNFa1b1c2-308*Gd5e1; H2G-TNFa6b5c1-308*Gd4e3; H3G-TNFa10b4c1-308*Gd4e3; H4G-TNFa11b4c1-308*Gd4e3; H5G-TNFa2b1c2-308*Gd4e1; H6G-TNFa7b4c1-308*Gd4e3; haplotypes associated with the *TNFA*-308*A allele were designated as follows: H1A-TNFa2b3c1-308*Ad2e3; H2A-TNFa4b5c1-308*Ad4e3; H3A-TNFa2b3c1-308*Ad1e3; H4A-TNFa4b5c1-308*Ad3e3; H5A-TNFa5b7c1-308*Ad5e3; H6A-TNFa3b5c1-308*Ad4e3; H7A-TNFa4b7c1-308*Ad4e3; H8A-TNFa2b1c2-308*Ad5e3.

Results

***TNFA*-308G>A SNP Case-Control Study.** Genotype frequencies of the *TNFA*-308G>A polymorphism in the control group did not deviate significantly from those

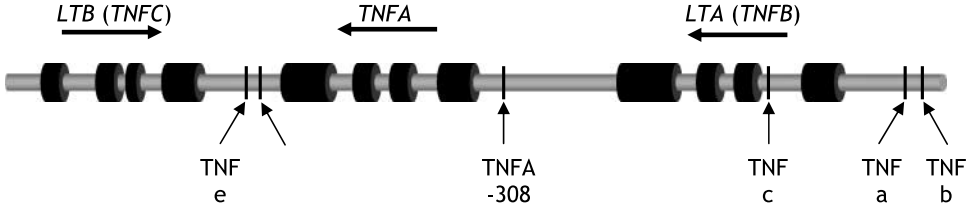

Figure 1. TNF gene cluster region with the location of the five microsatellites and the *TNFA* −308G>A polymorphism (adapted from Hajeer AH & Hutchinson IV 2001). Black blocks represent exons, thin longitudinal lines represent polymorphisms, and arrows represent the direction of transcription.

Table 1. Observed allelic frequencies of the *TNFA*–308*G>A SNP and TNF microsatellites in gastric carcinoma cases and healthy controls

Polymorphism	Alleles	Cases n = 508	Controls n = 713
TNFA –308	G	0.748	0.811
	A	0.252	0.189
TNF microsatellites		n = 122	n = 169
TNFa	a1	0.024	0.018
	a2	0.260	0.225
	a3	0.032	0.036
	a4	0.132	0.118
	a5	0.112	0.098
	a6	0.072	0.112
	a7	0.064	0.086
	a8	0.004	0.015
	a9	0.024	0.027
	a10	0.140	0.166
	a11	0.076	0.071
	a12	0.012	0.003
	a13	0.048	0.027
TNFb	b1	0.136	0.157
	b3	0.152	0.107
	b4	0.268	0.352
	b5	0.360	0.320
	b7	0.084	0.065
TNFc	c0	0.004	0
	c1	0.680	0.657
	c2	0.316	0.343
TNFd	d1	0.004	0.044
	d2	0.112	0.047
	d3	0.024	0.124
	d4	0.456	0.432
	d5	0.276	0.278
	d6	0.108	0.056
	d7	0.020	0.018
TNFe	e0	0.008	0
	e1	0.140	0.130
	e2	0.072	0.047
	e3	0.780	0.822

expected under Hardy-Weinberg equilibrium (Table 1). In this group, the -308*G allele had a frequency of 0.811 and the -308*A allele had a frequency of 0.189. In the gastric carcinoma group the frequencies of the -308*G allele and the -308*A alleles were, respectively, 0.748 and 0.252. Genotype frequencies among the gastric carcinoma cases and controls are summarized in Table 2. Both control groups have similar *TNFA*-308G>A genotype frequencies. The comparison of *TNFA*-308G>A genotype frequencies between cases and controls confirmed the existence of a significant association of the *TNFA*-308*A allele and the increased risk of gastric carcinoma with an OR of 1.7 (95% CI, 1.3-2.2; Table 1).

TNF Microsatellite Frequencies and Haplotypic Structure. The observed distributions for the TNFa, b, c, d, and e microsatellites are similar to those previously reported (13, 31). The TNFa microsatellite is the most polymorphic with 13 out of 14 known alleles present in our study population. TNFb, c, d, and e exhibited, respectively, 5, 3, 7, and 4 alleles. Table 1 shows the allelic frequencies at the five TNF microsatellite loci. TNF microsatellite alleles and the *TNFA*-308G>A SNP were phased into haplotypes by maximum likelihood. Haplotype inference disclosed a higher number of haplotypes associated with the *TNFA*-308*G allele than with the *TNFA*-308*A allele in both cases (75 and 22, respectively) and controls (84 and 19, respectively). This result supports the hypothesis that the *TNFA*-308*A allele is the most recent one. The frequencies of the *TNFA*-308*A–associated haplotypes are represented in Fig. 2. No significant differences could be observed relatively to the *TNFA*-308*G allele–associated haplotypes (designated H1G to H6G, Fig. 2). Regarding the *TNFA*-308*A allele–associated haplotypes (designated H1A to H8A), the most prominent difference was that found for the H1A haplotype, which was observed in 33.1% of the cases and in 12.5% of the controls. We have also found two other haplotypes (H7A and H8A) associated with the *TNFA*-308*A allele that are present only in the cases (although in very low frequencies) and three haplotypes present only in the control population (H3A, H4A, and H6A). A population differentiation test was performed showing that the control and the gastric carcinoma groups are significantly different ($P < 0.005$).

Discussion

It is well established that gastric carcinoma depends on the combined effects of *H. pylori* infection, environmental factors, and host susceptibility. The *TNFA*-308G>A promoter SNP is one of the most frequently reported candidate host factors associated with the risk for gastric carcinoma. However, the increasing number of published studies has revealed a conspicuous lack of reproducibility among the various populations (23, 24). So far, it is not known whether the *TNFA*-308G>A SNP is acting as an etiologic susceptibility locus or if it is associated in linkage disequilibrium with a causal sequence variant that has not yet been identified. In order to help clarify the association between the *TNFA*-308G>A polymorphism and the risk of gastric carcinoma, we used a haplotype-based approach by genotyping five microsatellites surrounding that SNP.

Although a similar strategy using the same markers has been used for the study of susceptibility to a number of conditions like Chagas' disease (12), pancreatitis (32), Crohn's disease (13), Alzheimer's disease (33), and rheumatoid arthritis (14), this study provides the first description of TNF microsatellite allele frequencies and derived haplotypes in the study of gastric carcinoma susceptibility. Moreover, our *TNFA*-308G>A SNP association study was based on a larger sample size than any of the previously published studies. Relatively to this SNP, we have confirmed the established association (22) between individuals carrying the *TNFA*-308*A allele and increased risk for developing gastric carcinoma (OR, 1.7; 95% CI, 1.3-2.2). In fact, two recent meta-analyses (23, 24) identify the -308G>A as the single *TNFA* SNP displaying

Table 2. Genotype distribution of the *TNFA*-308G>A polymorphism in gastric carcinoma cases and healthy controls

Genotypes	Cases n = 508	Controls n = 713	OR (95% CI)
GG	330 (65.0%)	544 (76.3%)	1 (referent)
A carrier	178 (35.0%)	169 (23.7%)	1.7 (1.3-2.2)

NOTE: P – value Hardy-Weinberg Equilibrium control population = 0.91.

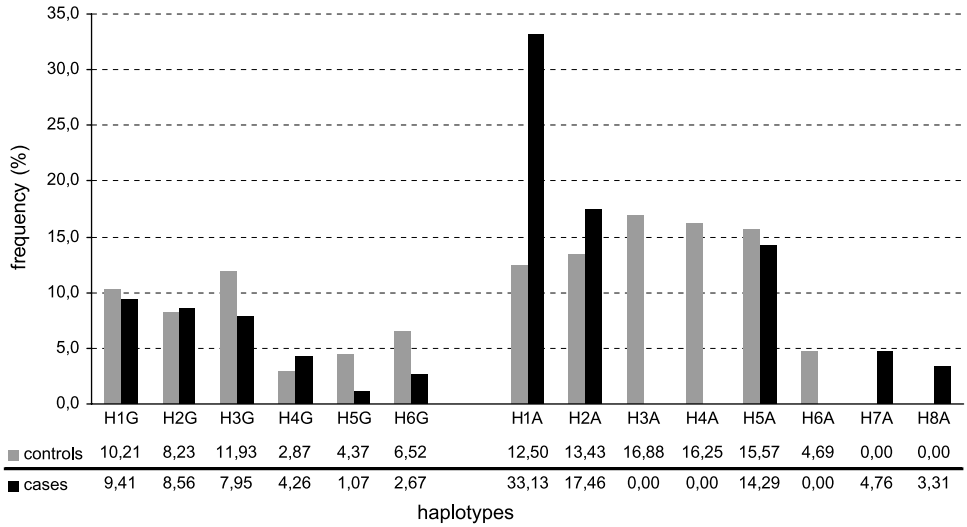

Figure 2. Distribution of the most frequent TNF microsatellite haplotypes associated with the *TNFA*-308*G and *A alleles. Haplotypes are described in Materials and Methods.

a statistically significant association with increased risk of gastric carcinoma. In those reports, evaluations of association with gastric carcinoma risk limited to anatomic and histologic subtypes or populations in Hardy-Weinberg equilibrium did not yield heterogeneous results and remained consistently strong. However, when studies considered Caucasian and Asian populations separately, the association was statistically significant only for the Caucasian. In their overall analysis, these studies did not rule out counter-examples in Caucasian populations like those reported by García-González et al. 2007 (34) and Kamangar et al. 2006 (35). The present understanding of the role of the *TNFA*-308G>A polymorphism on susceptibility to disease only emphasizes the importance of extending single-SNP association studies to haplotype-based approaches.

Our study shows that the *TNFA*-308*A–associated haplotypic structure is significantly different between cases and controls. Although the haplotype inference disclosed a higher number of haplotypes associated with the *G allele than with the *A allele in both cases and controls, no significant differences between groups could be observed in the *TNFA*-308*G haplotypic structure. In contrast, a population differentiation test showed that the control and gastric carcinoma groups are significantly different for the *TNFA*-308*A haplotypic structure as opposed to that of the *TNFA*-308*G. The most prominent difference was found for the H1A (TNFa2b3c1-308*Ad2e3) haplotype that showed a 3- fold increase in cases. We have also observed haplotypes associated with the *TNFA*-308*A allele that were found only in cases or controls. Taken together, these results suggest that the association between the promoter polymorphism and increased risk of gastric carcinoma is dependent on linkage disequilibrium. Thus, we cannot rule out the possibility that the TNF chromosomal region may contain a stronger genetic causal factor for gastric carcinoma development than any of the *TNFA* analyzed polymorphisms. The studies referred to above as using the TNF microsatellite haplotype-based approach have also found haplotypes that are more frequent in affected individuals than in controls, but none of those matched our susceptibility haplotypes.

In our search for a more suitable candidate gene and/or locus, we also did a TNF microsatellite single-allele association study (data not shown). Approximately 60% of the observed microsatellite alleles showed a higher frequency in the gastric carcinoma group than in the control group. Microsatellite markers were considered individually, and the association with gastric carcinoma risk was determined. We found higher magnitude associations between the TNF microsatellite markers and the increased risk of gastric carcinoma than that observed for the *TNFA*-308*A allele; however, only the TNFd2 allele was found to be significantly associated with the development of gastric carcinoma with an OR of 2.9 (95% CI, 1.4-5.7). We can conclude that the allele 2 of the TNFd microsatellite is also dependent on linkage disequilibrium and may be a better marker for increased risk of development of gastric carcinoma in our studied population.

In summary, the *TNFA*-308*A allele is associated with increased risk of gastric carcinoma development in our population. Additionally, we reported that this allele is in linkage disequilibrium with TNF microsatellite alleles and that a possible gastric carcinoma causal sequence variant is present in the same chromosomal region. This might represent a fundamental finding as it highlights the relevance of using haplotype-based approaches to provide a better understanding of susceptibility to gastric carcinoma and the consequent differences in incidence among populations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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