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### TNF-α Receptor 1 Promoter Gene Polymorphisms and Susceptibility to Gastric Cancer Related to H. Pylori Infection in Moroccan Population

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**Abstract:** Helicobacter pylori (H.pylori) infection is the main cause of serious gastric illness such as gastric cancer (GC). Single nucleotide polymorphisms (SNPs) that affect immune response genes may influence the host inflammatory reactions, then the development of diseases. To date, no reported studies investigated the relationship between Tumor Necrosis Factor receptor 1 (TNFR1) gene polymorphisms (-383, -580 and -609) and the development of gastric cancer. The aim of this study is to evaluate the involvement of TNFR1 SNPs in the development of chronic gastritis and GC, in Moroccan population. In total 265 patients and controls were selected for the study. Three polymorphisms of promoter gene TNFR1were researched in all subjects. The analysis of polymorphisms revealed the entire absence of TNFR1 -383 (A/G) SNP in our population, and no significant difference of allele distributions of TNFRSF1 -580 (A/G) was observed between GC, Chronic gastritis and Healthy Controls. While, the proportion of individuals carrying the TNFR1 -609 T allele was significantly lower in GC group than in controls group. These results suggest that the TNFR 1 -609 T allele has a protective function against GC.

Keywords: Gastric cancer, Helicobacter pylori, Tumor necrosis factor receptor 1, Single nucleotide polymorphisms.

#### 1. Introduction

Gastric cancer is an aggressive neoplasm that is associated with an extremely poor prognosis [1]. It is the fourth most commonly diagnosed cancer and the second common cause of cancer related mortality worldwide [2]. Its frequency varies greatly across different geographic locations, and has undergone significant changes in incidence over time. The highest incidences have been observed in Eastern Asia, Eastern Europe, and South America. However, the lowest incidence has been observed in North America and in most parts of Africa [3]-[4]-[5]. In Morocco, GC is the most common gastrointestinal cancer. It represents the fifth most common cancer; this is in concordance with that in developed countries [6].

GC develops through multiple steps. Pre-cancerous lesions of the stomach such as chronic atrophic gastritis, intestinal metaplasia (IM) and dysplasia preceding the development of GC [7]-[8].

Etiological factors are diverse and the major contributors in the pathogenesis of GC are *H. pylori* infection, host genetic variations and dietary factors. The most important is *H. pylori* infection; it is believed that roughly 65–80% of gastric cancers is associated with *H. pylori* infection [9]-[10]. In addition, several host genetic factors are important for the development of severe gastric immunopathologies and gastric cancer, it is shown that SNPs and/or points mutations in genes are affecting gastric acid secretion and immune response to *H. pylori* infection [11]-[12]. Genetic variations in cytokine genes can influence the inter-individual responses and disease susceptibility. The role of genes that encode pro- and anti-inflammatory cytokines and their receptors are well established in gastroduodenal diseases [13].

Host genetic factors are emerging as key determinants of disease risk for many cancers [14]. Many studies have investigated that polymorphisms in pro inflammatory cytokine genes, especially tumor necrosis factor alpha (TNF- $\alpha$ ) and its receptors, are associated with an increased risk of many cancer. Intracellular signaling and initiation of cellular response to TNF- $\alpha$  is provided by its two cell surface receptors, TNFR1 and TNFR2 [15]. Increased serum levels of TNF- $\alpha$  and its receptors have been described in patients with solid tumors, including oral carcinoma associated with adverse disease outcome [16]. Other studies have indicated a potential role of TNF- $\alpha$ , TNFR1 and TNFR2 in skin carcinogenesis [17]-[18].

The gene of TNFR1 has been mapped to chromosome12p13, and consists of 10 exons. The Analysis of this gene has identified multiple polymorphisms and the well-know SNPs were found in the promoter regions (-383, -580, - 609), exon 1 and in introns 2, 4, 6, 7 and 8 [19].

As TNFR1 gene polymorphisms in the promoter regions (-383, -580, - 609), have not been reported already in patients with chronic gastritis and gastric carcinoma. The present study was undertaken to assess the association between these SNPs at TNFR1 promoter sites, chronic gastritis and GC susceptibility for the first time in Moroccan population.

### 2. Materials and Methods

### 2.1. Patients and Controls

The study of TNFR1 polymorphisms has focused on patients with chronic gastritis, and patients with gastric cancer. The samples were collected within the Oncology Department at the IBN ROCHD University Hospital Center. Blood was collected in Ethylene Diamine Tetra Acetic Acid (EDTA) tube for genotyping TNFR1 polymorphisms, and the serum was collected in sec tube for diagnosis of *H. pylori* infection using Enzyme Linked Immunosorbent Assay (ELISA) (Human EL-HPYLG).

In total, 265 patients and controls were included in this study divided into three groups.  $1^{st}$  group compound of 94 patients with GC infected with *H. pylori* (mean age: 65±9 years; male/female ratio 45:49),  $2^{nd}$  group consisting of 97 patients with chronic gastritis infected with *H. pylori* (mean age: 49± 12 years; male/female ratio 46:51), and  $3^{d}$  group compound of 74 healthy controls group (HC), presenting no gastric disorders (mean age: 45±13 years; male/female ratio 36:38). All individuals participant were informed about their inclusion in the study and agreed to it in a writing form.

### 2.2. DNA extraction and quantification

The genomic DNA was extracted from the whole blood samples EDTA using commercially available kit (Invitrogen). The quantity and purity of DNA were checked by 1.0% agarose gel electrophoresis and also by the ratio of Optical Density (OD) at 260 nm and 280 nm using Nanodrope (NanoVue plus). Then it was stored at -20 °C until use.

### 2.3. Genotyping of TNFR1 polymorphisms

Genotyping of TNFR1 -609 (G/T), TNFR1-580 (A/G) and TNFR1 -383 (A/C) gene polymorphisms was performed by sequencing. Firstly, the TNFR1 gene (359 bp) was amplified from genomic DNA extracted from blood sample, by polymerase chain reaction (PCR), the PCR was carried out in a total reaction volume of 20 µL, consisting 400 ng genomic DNA, 200 µM of dNTP, 2.5 µM of primers as described by et (2001)[20]: Forward 5'-Allen al CGGACGCTTATCTATATCTC-3' (-706 to -687) and Reverse 5'-TTGTAGTCCAGTCACAAGCA-3' (-347 to -366), 1.5mM of MgCl<sub>2</sub>, and 2 unit of Taq polymerase in PCR reaction buffer at concentration of 1x.Thermo cycling conditions of PCR were as follows: initial denaturation at 94 °C for 10 minutes, followed by 35 cycles of 94 °C for 30 seconds, 57°C for 30 seconds, 72°C for 1 minute, and then a final extension at 72°C for 7 minutes.

The PCR products were analyzed by 1.5% agarose gel electrophoresis and purified using 0.5  $\mu$ L of exonuclease (Amersham pharmacia biote), 0.5  $\mu$ L alkaline phosphatase (Thermo Shrimp Alkaline Phosphatase, Amersham pharmacia biotech) in a final volume of 8  $\mu$ L, and 3-10 ng of PCR product and incubated at 37°C for 30min and 80°C for 15 min. DNA cycle sequencing was carried out in 10  $\mu$ L

reaction volume containing using 3  $\mu$ L of purified PCR product, 1  $\mu$ L of primer (3.2 pmol), 1  $\mu$ L of BigDye and 2  $\mu$ L of BigDye sequencing buffer «BigDye Terminator V3.1 » 5X . The forward and reverse sequencing primers were the same as those used in the PCR amplifications, in separate sequencing reactions. Cycling conditions for the sequencing of PCR products were 96°C for 2 min, 25 cycles of 96 °C for 10 seconds, 5 seconds at 50°C, and 4 min at 60°C. Residual dideoxy terminators were removed by ethanol precipitation and sequences were analyzed on an Applied Biosystems 377 DNA sequencer.

### 2.3. Statistical Analysis

Evidence for deviation from Hardy-Weinberg equilibrium of alleles at individual loci was assessed by exact tests. Differences in the proportion of TNFR1 -609 (G/T), TNFR1 -580 (A/G) and TNFRSF1-383 (A/C) and the genotype frequencies between cases and controls were assessed by test khi2 statistics. Crude odds ratios (ORs), adjusted ORs, and the simultaneous effect of the genetic polymorphisms with 95% confidence intervals (CIs) were estimated by logistic regression analysis. ORs and unconditional logistic regression models were computed using the program Epi Info 2002 (ftp://ftp.cdc.gov/pub/Software/epiinfo/). Differences were considered to be significant at a P value < 0.05. All statistical tests were 2-sided.

### 3. Results

Patients with diagnosed chronic gastritis and gastric cancer dues to *H. pylori* were investigated compared to healthy controls.

# **3.1.** Genotype frequencies distribution of TNFR1-609 (G/T), TNFR1-580 (A/G) and TNFR1 -383 (A/C) polymorphisms

The genotype distribution of the polymorphisms TNFR1 - 609 (G/T), and TNFR1 - 580 (A/G) were assessed with the Hardy-Weinberg equilibrium (HWE) for both patient and control groups [HWE pvalue > 0.05] (table 1 and 2).

Firstly, the study of TNFR1 polymorphisms was focused on the two most studied SNPs, -609 (G/T) and -383 (A/C). The sequences analysis has shown the complete absence of mutation at position -383 in our Moroccan population. In contrast, the frequency of TNFR1 -609 (G/T) SNPs occurrence in our population was 23.77%. TNFR1 -609 G/G homozygous was markedly higher in the GC group (73.41%) than in the HC group (58.1%) (Table2). For chronic gastritis group, the comparison of this genotype frequencies with healthy controls showed no significant difference [pvalue=0.77](table 1).

Secondly, the analysis of promoter sequences of TNFR1 from the position -706 to -347 had revealed the presence of mutation at position -580, characterized by the transition from adenine A to guanine G; this mutation has occurred in our population with a frequency of 15.1%. We noticed that the G/G homozygous allele was very rare, and no difference

was observed in the genotype distribution between groups [pvalue=0.96; pvalue=0.55](table 1).

 Table 1: Genotype and allele frequencies of TNFR1 - 580 (A/G) among patients /controls and association with chronic gastritis and gastric cancer

SNP	Healthy controls	Gastritis group			Gastric cancer group							
	n (%)	n (%)	$X^2$	P value	n (%)	$X^2$	P value					
TNFR1 -580 (A/G)												
AA	56 (75.7)	66 (68.04)			71(75.54)							
AG	15(20.3)	30 (30.92)	3.79	0.15 NS	19(20.21)	0	1 NS					
GG	3 (4)	1 (1.03)			4(4.25)							
Total	74	97			94							
Allele A	127 (85.81)	162 (83.5)			161(85.6)							
Allele G	21 (14.19)	32 (16.50)	0.34	0.55 NS	27(14.36)	0.002	0.96 NS					
Total	148	194			188							
HWE (P)	0.14 E	0.22 E			0.084E							

 Table 2: Genotype and allele frequencies of TNFR1 -609 (G/T) among patients/ controls and the association with chronic gastritis and gastric cancer

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SNP	Healthy	Gastritis group			Gastric cancer group						
	controls										
	n (%)	n (%)	$X^2$	P value	n (%)	$X^2$	P value				
TNFR1 -609 (G/T)											
GG	43 (58.10)	5 (52.57)			69 (73.41)						
GT	23 (31.09)	34 (35.06)	0.52	0.77 NS	21 (22.34)	5.15	0.07 NS				
TT	8 (10.81)	12 (12.37)			4 (4.25)						
Total	74	97			94						
Allele G	109 (73.65)	136 (70.1)			159 (84.5)		0.01 S				
Allele T	39 (26.35)	58 (29.9)	0.62	0.43 NS	29 (15.43)	6.12					
Total	148	194			188						
HWE (P)	0.08 E	0.10 E			0.16 E						

G/T: guanidine/thymine, A/G: adenine/guanine, HWE: Hardy-Weinberg equilibrium, E: population in equilibrium polymorphism, NS: not significant, S: significant.

# 3.2. Association of polymorphisms TNFR1 -580 (A/G) and -609 (G/T) with the occurrence of chronic gastritis and gastric cancer

A significant difference of TNFR1 (G/T) allele distributions was observed between GC group and HC. Furthermore, G allele had a greater risk for GC compared to T allele [OR=0.5098; IC: 0.29-0.87; p value=0.01] (table 2), while no correlation was observed between TNFR1 -609 (G/T) and chronic gastritis [OR= 1.21; IC:0.75-1.95; pvalue=0.43] (table 2).

The association between TNFRSF1-580 A/G polymorphism and susceptibility to chronic gastritis and GC had meaningless [OR= 1.19; CI: 0.65 - 2.17; pvalue=0.55]; [OR=1.01; IC: 0.54- 1.87; pvalue=0.96], respectively (table 1).

### 4. Discussion

Tumor necrosis factor alpha (TNF- $\alpha$ ) is a pro-inflammatory cytokine produced by activated macrophages; it executes its multiple biological functions through binding to its two cell surface receptors, TNFR1 and TNFR2. It is well known that TNF- $\alpha$  and its superfamily members have both beneficial and harmful activities, playing a role as a "double-edged sword"

[21]. Although TNF- $\alpha$  was discovered as a cytokine that could kill tumor cells, it is now clear that TNF  $\alpha$  can also contribute to tumorigenesis by mediating the proliferation, invasion and metastasis of tumor cells [22]. Polymorphisms in cytokines and receptor genes of immune system can influence the response to infectious agents, such as *H. pylori*. It may contribute to modulation of the gastric carcinogenesis risk [21][23]-[24]. A recent study of our team laboratory, has demonstrated the association between TNF-  $\alpha$  –238 (G/G) genotype and high risk of GC, and has identified a new polymorphism located in the promoter region at position –193, founded related with high risk of ulcer [25].

To our knowledge, to date, no reported studies investigated the correlation between TNFR1 gene polymorphisms and the development of severe gastric pathologies. Therefore, in this work we studied the association between TNFR1 polymorphisms and the risk of gastric diseases development. This is the first study on Moroccan population that checked polymorphisms in TNFRSF1 gene, which may contribute to different outcomes of infection and cause the development of gastric cancer.

The dysregulation of gene expression in the TNF-TNFR has been reported to be involved in the development and prognosis of various human cancers, including non-Small

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cell lung cancer [22]-[26]-[27] and breast cancer [28][29]. The abnormal expression of TNF-TNFR has been observed to be involved in the pathogenesis and treatment outcomes of various malignant tumors [26]-[28]. In case of skin cancer, the absence of TNFR1 or TNFR2 inhibited significantly tumor development [17]-[18]. Whereas, in a liver tumor model, it was suggested that carcinogenesis only depends to TNFR1 expression [30]. Moreover, in breast cancer cell lines, blocking TNFR1 or TNFR2 with specific antibodies impairs tumor survival signaling and the biological function of TNF  $\alpha$  [29]. In this study, we have chosen to sequence TNFR1 gene at the promoter site from the position -347 to -706, because this region composes three polymorphisms at -609(G/T), -580 (A/G) and -383 (A/C) positions.

The results of our study showed the complete absence of mutation at position -383 in our population. This mutation is characterized by its rarity, and our result is corroborated by other studies. In Chinese population the frequency of - 383 C allele was in the order of 0.07[31]; and 0.003[32] or completely absent in Caucasian population; In African Americans with rheumatoid arthritis the frequency was in order of 0.09[33].

We found no significant correlation of TNFR1 -580 (A/G) SNP with gastritis and GC, then [OR= 1.19; CI: 0.65 - 2.17; pvalue=0.55]; [OR=1.01; IC: 0.54-1.87; pvalue=0.96]. This mutation was observed has no influence on susceptibility to rheumatoid arthritis in African Americans [33].

The present study showed a significant difference of allele distributions of TNFR1-609 (G/T) was observed between GC group and HC. The proportion of individuals carrying the TNFR1 -609 T allele was significantly lower in GC group than in controls group [OR=0.5098; IC: 0.29-0.87; p value=0.01], and the -609 T/T homozygote was lower in patients with GC compared to HC. It seems, therefore, that -609 G/T TNFR1 SNP has a protective effect against GC. This result is in concordance with another study about oral cancer tumor model, in Indians population, that showed an interesting highly significant negative association between oral cancer tumor and TNFR1 -609 G/T [34]. TNFR1-609 was also observed associated with reduced risk of colon cancer [35] and invasive pulmonary aspergillosis [36].

The results of our study demonstrate that TNFR1 -609 G/T may present a protective mutation, these results can be explained on one hand by the fact that TNFR1 -609 (G/T) SNP causes an increase in the expression of TNFR1 on the cell membrane, and a decrease in the concentration of soluble TNFR1 (sTNFR1), consequently, enhances TNF-α activity against malignant cells. This hypothesis is reinforced by another study which examined the effect of the polymorphisms in TNFR genes on the expression levels of Membrane-Bound Type I and Type II Receptors, they proved that Homozygous TT individuals at SNP -609G/T TNFRI had a lower levels of sTNFRI compared to GG genotype carriers [37]. On the other hand, we can suppose that this mutation can enhance the death pathway and impairs the survival pathway NFKB of TNFR1. Therefore, we can conclude that TNFR1 plays an important role in carcinogenesis. So TNFR1 polymorphisms may be meaningful in the pathology of GC and may provide valuable prognostic information for patients at risk for developing gastric cancer

### 5. Conclusion

The proportion of individuals carrying the TNFR1 -609 T allele was significantly lower in GC group than in controls. Then, the frequency of G alleles was significantly higher in patients compared to controls. These results suggest that the TNFR1 -609 T allele has a protective function against GC, while -609 G allele increased the risk of GC development.

### 6. Future Scope

More advanced studies on the production and signaling pathway of TNFR1 are needed to elucidate the functionality of this SNP which can be the target of new therapeutic strategies.

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