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Haplotype Analysis of *TNFA* Gene in Peptic Ulcer Patients

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ABSTRACT The *TNFA* gene product TNF-α is a cytokine promoting an immune response after bacterial infections. An excessive production of the cytokine is thought to be connected with *Helicobacter pylori*-induced disorders like gastritis, peptic ulcer disease (PUD), intestinal metaplasia, or even gastric cancer. The synthesis of TNF-á is controlled at transcriptional level and dependent on single nucleotide polymorphisms (SNPs) of *TNFA* promoter region. SNPs assembled in haplotype have been implicated as potential risk factors for various autoimmune and infectious diseases. The aim of this study was to determine the frequencies of *TNFA* haplotypes composed of the four common single-nucleotide polymorphisms of the gene (-1031C>T: rs1799964, -863C>A: rs1800630, -857C>T: rs1799724, -308G>A: rs1800629) in peptic ulcer patients and to assess the significance of the haplotypes as the risk factors of *H. pylori* infection development. 203 peptic ulcer patients were genotyped using polymerase chain reaction-restriction fragment length polymorphism method. Haplotypes and degree of linkage disequilibrium (LD) were inferred with PHASE 2.1 and EMLD software. There was no statistically significant difference in haplotype frequencies between the *H. pylori*-infected and -uninfected peptic ulcer cases (p=0.62). Analogous association was also absent in the subgroups: peptic ulcer woman (p=0.69), peptic ulcer men (p=0.17). The locus pairs -308_-857, -863_-1031, and -857_-1031 was found to be in very strong LD , -308_-1031 and -857 and -863 in strong LD, and -308_-863 - in modest LD. *TNFA* haplotype structure is not connected with individual differences in susceptibility to development of *H. pylori* infection in peptic ulcer patients.

INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) is a cytokine known for having the multitude of functions. One of the most prominent is to promote of immune response after bacterial infections. In gastric mucosa infected by *Helicobacter pylori* TNF-α prompts recruitment of mononuclear cells and neutrophils into the gastric lamina propria (Crabtree 1991; Hüseyinov 1999). An excessive production of the cytokine could also exert some pathogenic effects (Beutler 1993) and is thought to be connected with *H. pylori*-induced disorders like gastritis, peptic ulcer disease (PUD), intestinal metaplasia, or even gastric cancer (Goto 2003; Shanks 2009).

The synthesis of TNF-α is controlled at transcriptional level in a cell type- and stimulus-specific manner involving different transcription factors to a promoter region of the *TNFA* encoding TNF-α (Falvo 2010). An important role in the regulation of *TNFA* expression is attributed to single nucleotide polymorphisms (SNPs) of promoter region of the gene. Accordingly, these genetic variations are implicated as potential risk factors for various autoimmune and infectious diseases.

To date, SNPs at positions -238, -308, -857, -863, -1031 of *TNFA* have been investigated in various populations afflicted by PUD or other *H. pylori*-induced disorders (Lee 2005; Lu 2005; Lee 2004; Wilschanski 2007; Kim 2006; Machado 2003). Unfortunately, studies focused on determining the importance of individual *TNFA* loci have produced non-significant or conflicting results. However, some evidence has supported the hypothesis that effects exerts by these SNPs could be observed or are more pronounced when the polymorphisms are researched and analysed concomitantly. Studies by Lu et al. (2005) conducted in Taiwanese revealed that risk of ulceration after *H. pylori* infection was higher for carriers of both -1031C or -863A allele of *TNFA* than for individuals having only one of the mentioned alleles. Similarly, the risk of gastric ulcers among Japanese was found to be greatest in simultaneous carriers of so called 'high-producer' alleles of *TNFA* -857/-863/-1031 than when the presence of these particular alleles was stated individually (Sugimoto 2007).

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Some research results could imply the best approach to investigate the relevancy of *TNFA* variations, in the development of gastroduodenal diseases, is to explore haplotype structure of *TNFA* gene. Although Chakravorty et al. (2008) did not stated any connection between single *TNFA* loci (-308, -857, -863, -1031) and *H. pylori*-mediated duodenal ulcer, the researchers found the *TNFA* haplotype -308G_-857C_-863A_-1031T in *H. pylori*-infected duodenal ulcer individuals was significantly more frequent than in the infected but ulcer-free cases. Thus far, haplotype analysis of the gene has been applied in the studies on type 1 diabetes (Stayoussef 2010), gastric cancer (Canedo 2008), iron deficiency anemia (Atkinson 2008), Behcet's disease (Park 2006), schizophrenia (Saviouk 2005), juvenile oligoarthritis (Zeggini 2002), hepatitis C virus infection (Thio 2004), and some others.

Recently, the researchers investigated association between individual -308G>A or -1031T>C SNPs of *TNFA* and peptic ulcer risk in Poles (Salagacka 2014). In this study the researchers conducted haplotype analysis of *TNFA* combining mentioned genotyping data and newly obtained for -308 locus and two further -857 and -863 loci of *TNFA*. The aim of presented research was to estimate the *TNFA* haplotype structure and its significance in development peptic ulcer disease in Polish population.

METHODOLOGY

The investigated group consisted of 203 patients diagnosed the first time as suffering from peptic ulcers (130 women and 73 men). Mean age of women was 52 years (minimum 14 years, maximum 85), and mean age of men was 53.6 years (minimum 20 years, maximum 84). All were diagnosed in the Department of Surgery, District Hospital, Leczyca, Poland. Patients who were treated with non-steroidal anti-inflammatory drugs were excluded. Data concerning exposure to carcinogens in patients and controls were not available. The investigation was in accordance with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of Medical University of Lodz. All subjects included in the study gave informed consent.

Helicobacter pylori infection was diagnosed at the time of gastroduodenoscopy using rapid urease test (*Instytut Zuwnosci i Zywienia*, Warsaw, Poland). The basis of the test is the ability of *H. pylori* to secrete the urease enzyme, which catalyzes the conversion of urea to ammonia and bicarbonate. From all investigated patients mucosa samples were taken during biopsy from the antrum of the stomach and placed into a medium containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium and changes the color of the specimen from yellow (negative) to red (positive).

DNA was isolated according to "Genomic DNA Prep Plus" protocol (*AandA Biotechnology*, Gdynia, Poland) from material biopsy specimens of gastric membrane mucosa. The purity and concentration of DNA samples were estimated spectrophotometrically. The samples were stored at -20^{0}C until analysis.

Genotyping was conducted using polymerase chain reaction-restriction fragment length polymorphism method. PCR mixture consisted of DNA template, 0.2 mM of each primer, 10µl of Jump Start RedTaq ReadyMix™ (*Sigma Aldrich*, Germany) and PCR-grade water to a final volume of 20 µl. Negative controls were included in each experiment (samples without DNA template). The primer sequences and PCR reaction conditions used were published earlier (Skoog 1999). Amplified DNA fragments were digested by restriction enzyme *Tai*I during 16 h at 65°C for -857C>T and at 37°C for –863C>A. DNA fragments generated by digestion were separated in 12% polyacrylamide gel. Electrophoresis pattern showed 106 and 22 bp fragments for wild type -857CC homozygote, 128 bp fragment for mutant -857TT homozygote and three bands 128, 106, and 22 bp fragments for -857CT heterozygote. 125 bp band for wild type -863CC homozygote, 101, and 24 bp bands for mutant -863AA homozygote, and 125, 101, 24 bp bands for -863CA heterozygote were present. PCR reaction products were separated in 2% agarose gel.

Haplotypes were statistically inferred using the PHASE v. 2.1 software. The PHASE program implements a Bayesian statistical method for reconstructing haplotypes from population genotype data (Stephens et al. 2001; Stephens and Donnelly 2003). A permutation test was performed to compare haplotype frequencies between populations. P-values <0.05 were assumed as significant in all tests conducted.

Linkage disequilibrium (LD) was estimated using EMLD software with the Expectation-

Maximization algorithm (Slatkin and Excoffier 1996). The degree of linkage disequilibrium was determined by using two standard measures: Lewontin's D' and the correlation coefficient.

RESULTS

Haplotype analysis presented in this paper was carried using data about *TNFA* SNPs obtained in 203 peptic ulcer Poles. Each of four investigated SNPs was analysed separately. Genotyping was successfully conducted in all 203 cases for -857 and -863 loci of *TNFA*, 202 cases for -308 locus, and 178 cases for -1031 locus. Data about -308G>A and -1031T>C SNPs of *TNFA* in 177 of 203 cases was presented earlier (Salagacka 2014). Genotyping data for all investigated polymorphisms was summarized in Table 1.

In the investigated group of peptic ulcer patients 13 possible haplotypes were inferred (Table 2). The $T_{-1031}C_{-863}C_{-857}G_{-308}$ haplotype was of predominant frequency (52.3%), the three others $T_{-1031}C_{-863}C_{-857}A_{-308}$, $C_{-1031}A_{-863}C_{-857}G_{-308}$, $T_{-1031}C_{-863}T_{-857}G_{-308}$ were of similar frequency (16.0%, 12.8%, 11.5%, respectively). The frequency of the rest inferred haplotypes except $C_{-1031}C_{-863}C_{-857}G_{-308}$ (5.4%) was estimated to be under 1%.

Using rapid resase test, the *H. pylori* infection was stated in 101 examined patients. In the rest 102 individuals result of the test was negative. To assess the significance of *TNFA* haplotype structure to *Helicobacter pylori* infection development in peptic ulcer patients, the haplotype frequencies were estimated and then compared in subgroups of *H. pylori*- infected and *H. pylori*-uninfected peptic ulcer cases. The frequencies in both subgroups were similar to these obtained in the whole peptic ulcer and did not differ significantly between the subgroups (p=0.62).

In the next step, the whole investigated peptic ulcer cohort was divided according to gender and haplotype structure of *TNFA* was inferred separately in subgroup of women and men. Among the peptic ulcer women frequency of estimated *TNFA* haplotypes in a sub-group of *H. pylori*-infected cases was nearly the same as in the subgroup of *H. pylori*-uninfected cases (Table 2, p=0.69).

In the peptic ulcer men, some differences in particular *TNFA* haplotype frequencies between subgroups of *H. pylori*-infected and -uninfect-

Table 1: Gentotyping result for all investigated *TNFA* polymorphisms

		Peptic ulcer patients			*Peptic ulcer women*			*Peptic ulcer men*		
		All	*H. pylori-uninfected*	*H. pylori-infected*	*All*	*H. pylori-uninfected*	*H. pylori-infected*	*All*	*H. pylori-uninfected*	*H. pylori-infected*
-308	GG	137 (67)8%	69 (68)3%	68 (67)3%	85 (65)9%	46 (70)8%	39 (60)9%	52 (71)2%	23 (63)9%	29 (78)4%
	GA	62 (30)7%	30 (29)7%	32 (31)7%	42 (32)6%	18 (27)7%	24 (37)5%	20 (27)4%	12 (33)3%	8 (21.6%)
	AA	3 (1.5%)	2 (2.0%)	1 (1.0%)	2 (1.6%)	1 (1.5%)	1 (1.6%)	1 (1.4%)	1 (2.8%)	0 (0.0%)
-857	CC	158 (77)8%	75 (74)3%	83 (81)4%	108 (83)1%	53 (81)5%	55 (84)6%	50 (68)5%	22 (61)1%	28 (75)7%
	CT	40 (19)7%	23 (22)8%	17 (16)7%	19 (14)6%	10 (15)4%	9 (13.8%)	21 (28)8%	13 (36)1%	8 (21.6%)
	TT	5 (2.5%)	3 (3.0%)	2 (2.0%)	3 (2.3%)	2 (3.1%)	1 (1.5%)	2 (2.7%)	1 (2.8%)	1 (2.7%)
-863	CC	149 (73)4%	78 (77)2%	71 (69)6%	92 (70)8%	48 (73)8%	44 (67)7%	57 (78)1%	30 (83)3%	27 (73)0%
	CA	50 (24)6%	22 (21)8%	28 (27)5%	34 (26)2%	16 (24)6%	18 (27)7%	16 (21)9%	6 (16.7%)	10 (27)0%
	AA	4 (2.0%)	1 (1.0%)	3 (2.9%)	4 (3.1%)	1 (1.5%)	3 (4.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
-1031	TT	113 (63)5%	59 (64)1%	54 (62)8%	68 (60)7%	35 (60)3%	33 (61)1%	45 (68)2%	24 (70)6%	21 (65)6%
	CT	62 (34)8%	31 (33)7%	31 (36)0%	42 (37)5%	22 (37)9%	20 (37)0%	20 (30)3%	9 (26.5%)	11(34.4%)
	CC	3 (1.7%)	2 (2.2%)	1 (1.2%)	2 (1.8%)	1 (1.7%)	1 (1.9%)	1 (1.5%)	1 (2.9%)	0 (0.0%)

Table 2: Comparison of estimated *TNFA* haplotype frequencies (-1031_-863_-857_-308) in *H. pylori*-infected and -uninfected peptic ulcer patients

	Peptic ulcer patients						*p-value (permutation test)*
	all (n=203)		*H. pylori-uninfected (n=101)*		*H pylori-infected (n=102)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
T_C_C_G	0.5230	0.0097	0.4999	0.0108	0.5406	0.0114	0.62
T_C_C_A	0.1597	0.0084	0.1707	0.0089	0.1487	0.0101	
C_A_C_G	0.1279	0.0081	0.1027	0.0078	0.1531	0.0097	
T_C_T_G	0.1151	0.0033	0.1350	0.0045	0.0952	0.0036	
C_C_C_G	0.0540	0.0045	0.0722	0.0052	0.0357	0.0070	
T_A_C_G	0.0049	0.0033	0.0062	0.0024	0.0035	0.0048	
T_A_T_G	0.0045	0.0020	0.0046	0.0023	0.0045	0.0021	
C_A_C_A	0.0042	0.0063	0.0040	0.0066	0.0044	0.0070	
C_C_C_A	0.0035	0.0033	0.0016	0.0029	0.0054	0.0052	
C_A_T_G	0.0011	0.0026	0.0010	0.0026	0.0011	0.0029	
C_C_T_G	0.0010	0.0018	0.0006	0.0017	0.0015	0.0027	
T_C_T_A	0.0010	0.0022	0.0015	0.0033	0.0006	0.0018	
T_A_C_A	0.0000	0.0005	0.0000	0.0005	0.0000	0.0005	
	Peptic ulcer women						
	all (n=130)		*H. pylori-uninfected (n=65)*		*H pylori-infected (n=65)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
T_C_C_G	0.5377	0.0158	0.5381	0.0183	0.5372	0.0165	0.69
T_C_C_A	0.1610	0.0133	0.1523	0.0157	0.1698	0.0139	
C_A_C_G	0.1339	0.0141	0.1074	0.0162	0.1608	0.0147	
T_C_T_G	0.0846	0.0038	0.0978	0.0041	0.0713	0.0060	
C_C_C_G	0.0483	0.0055	0.0705	0.0067	0.0258	0.0083	
C_A_C_A	0.0114	0.0112	0.0132	0.0139	0.0097	0.0107	
T_A_T_G	0.0076	0.0016	0.0074	0.0019	0.0077	0.0023	
T_A_C_G	0.0070	0.0038	0.0089	0.0031	0.0050	0.0066	
C_C_C_A	0.0044	0.0039	0.0023	0.0039	0.0066	0.0058	
C_C_T_G	0.0019	0.0028	0.0002	0.0011	0.0036	0.0053	
T_C_T_A	0.0009	0.0017	0.0008	0.0023	0.0011	0.0027	
C_A_T_G	0.0008	0.0030	0.0007	0.0026	0.0009	0.0037	
T_A_C_A	0.0005	0.0014	0.0005	0.0018	0.0005	0.0018	
	Peptic ulcer men						
	all (n=73)		*H. pylori-uninfected (n=36)*		*H pylori-infected (n=37)*		
	Frequency	*S.E.*	*Frequency*	*S.E.*	*Frequency*	*S.E.*	
T_C_C_G	0.5094	0.0087	0.4450	0.0086	0.5721	0.0130	0.17
T_C_T_G	0.1696	0.0039	0.2057	0.0068	0.1346	0.0027	
T_C_C_A	0.1489	0.0050	0.1925	0.0063	0.1066	0.0054	
C_A_C_G	0.1085	0.0035	0.0829	0.0024	0.1333	0.0055	
C_C_C_G	0.0609	0.0071	0.0710	0.0058	0.0511	0.0120	
T_C_T_A	0.0011	0.0035	0.0017	0.0058	0.0006	0.0027	
C_A_C_A	0.0006	0.0030	0.0003	0.0020	0.0008	0.0044	
T_A_C_G	0.0004	0.0017	0.0000	0.0002	0.0009	0.0033	
C_C_T_G	0.0004	0.0017	0.0008	0.0034	0.0000	0.0000	
C_A_T_G	0.0001	0.0007	0.0001	0.0014	0.0000	0.0000	

ed cases were stated (Table 2). Among infected men the $T_{-1031}C_{-863}C_{-857}G_{-308}$ and $C_{-1031}A_{-863}C_{-857}G_{-308}$ haplotypes were estimated to be more frequent in infected than in uninfected men (75.1% and 13.3% vs. 44.5% and 8.3%, respectively). Contrarily, the $T_{-1031}C_{-863}T_{-857}G_{-308}$ and $T_{-1031}C_{-863}C_{-857}A_{-308}$ were assessed to be more frequent in uninfected than in infected men (20.6% and 19.2% vs. 13.3% and 10.7%, respectively). However, the stated differences were not statistically significant (p=0.17).

Lastly, the pair-wise linkage disequilibrium was estimated in the whole investigated peptic ulcer cohort. Calculated Lewontin's D' and correlation coefficients between pairs of loci are summarized in Table 3. The locus pair -308_-857, -863_-1031, and -857_-1031 was found to be in very strong LD (D' form 0.9999 to 0.8901). The LD between -308 and -1031 loci, and between -857 and -863 loci was strong (D' 0.4633 and 0.4940, respectively), and only -308_-863 locus pair was in modest LD (D' 0.1336).

DISCUSSION

Single nucleotide polymorphisms are most common genetic variation in the human genome. In recent years, SNPs are widely researched to find the susceptibility genes for complex diseases. As an individual SNP has relatively low information content, the approach relies on analysis genetic markers could be insufficient in some cases. To overcome this limitation, haplotype analysis is used. To date, it was successfully utilized in genetic association studies, searching for the genes causing complex diseases, predicting the severity and prognosis of certain genetic diseases of Mendelian inheritance (Lee 2005).

Currently, many polymorphisms of genes taking part in inflammation process and immunological response to *H. pylori* are researched now to establish genetic risk factors of development of PUD and other gastric diseases (gastritis, intestinal metaplasia, or gastric cancer). These are, for example, genes encoding interleukins (*IL1B, IL2, IL4, IL6, IL8, IL10*), toll-like receptor 4 (*TLR4*), or mannose-binding-2 lectin (*MBL2*). Among them there is also *TNFA* gene encoding Tumor Necrosis Factor-alpha (TNF-α) (Shanks 2009; Sugimoto 2010; Hishida 2010). In the presented research, the researchers analyzed haplotype structure of the *TNFA* and its significance in development peptic ulcer disease in Polish population.

In the investigated peptic ulcer cohort five *TNFA* haplotypes with frequencies exceeding 5% accounted for above 99% of all inferred haplotypes. This result is very similar to that obtained earlier by Ploski et al. (2004), who carried out *TNFA* haplotype analysis in unrelated Polish healthy subjects. The comparable haplotype frequencies were also stated in healthy UK Caucasians (Anglo-Saxon) (Zeggini 2002). Considering this, it could be speculated that there is no association between *TNFA* haplotype structure and susceptibility to peptic ulcer disease in Polish population. However, it should be confirmed in further research.

The researchers found the virtually complete linkage disequilibrium between -308 and -857, and -857 and -1031 loci. Also, the -863_- 1031 locus pair showed very strong LD. It partially concordant with results obtained earlier (Ploski 2004; Higuchi 1998) that -1031 and -863 loci are in strongly interdependent. Also in the research conducted by Zeggini et al. (2004) the -863A allele was found exclusively on haplotypes including -1031C.

It is known that genetic background of the host is a significant factor contributing to the outcome of chronic virus and bacterial infections. The active balance between the host immune defense mechanisms and growth of pathogens which determines the course of the infection is influenced by the TNF-α. *TNFA* SNPs were stated to by connected with susceptibility and/or progression of several infectious diseases, like leprosy, malaria, chronic B and C hepatitis (Bayley 2004), and also *H. pylori*-induced disorders (Wilschanski 2007; Lee 2005; Lee 2005; Kim 2006).

The researchers did not find any differences in *TNFA* haplotype frequencies between *H. pylori*-infected and *H. pylori*-uninfected peptic ulcer cases, so it could be stated that genomic structure of the *TNFA* do not influence the risk of development of the PUD. It states in contradiction with some previously published results. For example, Chakravorty et al. (2008) observed an association between *TNFA* $G_{-308}C_{-857}A_{-863}T_{-1031}$ haplotype and the presence of duodenal ulcers in *H. pylori*-infected individuals from eastern Indian population. In Spanish Caucasian haplotype named TNF-I *H. pylori*-infected carriers were found to have an increased risk of peptic ulceration (Lanas 2001). However, the mentioned

results cannot be fully comparable to these obtained by us. In the presented research peptic and duodenal ulcer cases are analyzed as a one peptic ulcer group, differently than in the investigation of Chakravorty et al. (2008), which concerns only duodenal ulcer cases. In the mentioned above paper published by Lanas et al. (2001) the analyzed haplotypes comprised not only *TNFA* but also loci of the neighbouring *LTA* gene encoding lymphotoxim-α, other inflammatory cytokine. Considering this, it can be assumed that some particular combination of different cytokines is important to development of different clinical outcomes. In Spanish Caucasians *TNFA* -308 G>A and -238G>A and *LTA Nco*I and *Bse*I polymorphisms analyzed individually or in combinations were not influenced on the susceptibility to duodenal ulcers, but simultaneous presence of their 'low producing' alleles and IL-1RN*2/IL-1B-31T/IL-1B-511/IL-1B+3954 alleles was increased in *H. pylori*-positive duodenal ulcers in comparison to *H. pylori*-positive healthy individuals (Garcia-Gonzalez 2005). To confirm this hypothesis, a larger number of polymorphic loci should be investigated in the future in our peptic ulcer patients.

CONCLUSION

There is the virtually complete linkage disequilibrium between -308 and -857, and -857 and -1031 loci. In the investigated peptic ulcer patients' haplotype structure of *TNFA* gene is not responsible for individual differences in predisposition to *H. pylori* infection development.

However, further analysis conducted in a lager cohort of patients concerning additional genetic loci are needed.

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